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The role of integrins in early colonic cancer liver metastases development

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DECLARATION CONCERNING THESIS PRESENTED FOR THE DEGREE OF

DOCTOR OF MEDICINE

I, **JOHN HENRY PHILIP ROBERTSON**

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solemnly and sincerely declare, in relation to the thesis entitled:

The role of integrins in early colonic cancer liver metastases development

(a) That work was done by me personally

and (b) The material has not previously been accepted in whole, or in part, for any other degree or diploma.

Signature: Date: 19th Feb '08

Dedication

I would like to express my sincere gratitude to all those who have helped me during my research period.

Firstly, I would like to thank my wife, Laura and my family for their continued support over the last two years.

Secondly, I would like to express my gratitude to the Academic Department of Surgery Unit, Royal Free Hospital, London for all their assistance during my M.D. In particular I would like to thank Professor Marc Winslet, Professor Alexander Seifalian, Dr Kevin Sales and Dr Shi Yu Yang for patiently listening to all my ideas and constantly steering me in the correct direction. I would also like to express a special thanks to both my colleague Mr Arthur Iga who has helped me during most of the experimental procedures and the late Dr Wenxuan Yang who was instrumental in my MD concept and the initial planning of my thesis.

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Abstract

Annually, almost one million cases of colorectal cancer are diagnosed and almost half a million deaths are attributed to this disease worldwide. The liver is the most common and critical site of distant metastasis. The primary locus of the colorectal tumour can frequently be managed by surgery alone or with neoadjuvant and adjuvant oncological therapy. Management of the metastatic spread is more difficult.

For more effective oncological management of disseminated colorectal cancer, therapies must be devised that target the different individual stages of metastasis development. This theory is supported by the survival benefit shown using Bevacizumab, a selective inhibitor of Vascular Endothelial Growth Factor, as an adjuvant to standard chemotherapy for metastatic colorectal cancer.

Recent work showed that specific integrin subunits α2, α6 and β4 played a role *in vivo* in human HT29 colorectal cancer cell migration and extravasation. Through the techniques of Immuncytochemistry and Western Blotting, these three integrins were shown to be expressed not only in the control HT29 cell line but also in the unrelated rat DHDK12 colonic cancer cell line – a novel finding. Functional blocking by antibodies of these integrin subunits, in the rat DHDK12 cell line, was studied *in vivo* using intravital video microscopy. This produced a significant reduction in tumour cell extravasation compared to control groups – wild type and wild type with non specific IgG. Serial measurements were used and then compared using ANOVA with Bonferroni's Multiple Comparison.

Analysis of current established *in vivo* models of early colorectal liver metastasis development were performed using the intravital video microscopy. The results showed colorectal cancer cell line-host selection and biological compatibility had a significant effect on early metastasis development. The *in vivo* model of BDIX rat with DHDK12 cell provides a highly biologically accurate system to allow the study of the early events of colonic cancer liver metastasis.

In conclusion, integrins play an important role in the early development of colonic cancer cell liver metastasis. Functional blocking of integrin subunits α2, α6 and β4, expressed in unrelated colorectal cancer strains, reduce colonic cancer cell migration. Colorectal cancer cell line-host selection and biological compatibility had a significant effect on early colorectal liver metastasis establishment.

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Hypothesis

The hypothesis has two components:

- The integrin subunits α2, α6 and β4 play a key role in human colorectal cancer cell migration and extravasation. These subunits may have a key role in early colorectal liver metastasis development from other colorectal cancer cells lines.

- Current *in vivo* models of early colorectal cancer liver metastasis use colorectal cancer cell line-host combinations which have a low biological compatibility. Colorectal cancer cell line-host selection may have a significant effect on early metastasis development.

TABLE OF CONTENTS

LIST OF FIGURES

List of Tables

List of abbreviations

DMEM Dolbecco's Modified Eagle Media

ECACC European Collection of Cell Cultures Media

FBS Fetal Bovine Serum

FITC fluorescein isothiocyanate

GFP Green Fluorescent Protein

HR Heart rate

IgG Immunoglobulin

IVVM Intravital videomicroscopy

MABP Mean arterial blood pressure

SaO_2 Oxygen saturation

SD Sprague Dawley Rat

Temp Temperature

VEGF Vascular Endothelial Growth Factor

WT Wild Type

CHAPTER 1

INTRODUCTION & AIMS

1.1 Thesis Outline

Colorectal cancer was the second most common cancer diagnosed in Europe in 2004 with 203,700 new cases diagnosed (Boyle and Ferlay, 2005). Each year worldwide, almost one million cases of colorectal cancer are diagnosed and almost half a million deaths are attributed to this disease (Parkin *et al.*, 2001). The liver is the most common and critical site of distant metastasis. At diagnosis, as many as 25% of colorectal cancer patients have established liver metastases (Kavolius *et al.*, 1996).

The primary locus of the colorectal tumour is frequently managed by surgery either alone or in combination with neoadjuvant and adjuvant oncological therapy. Management of the metastatic spread is more difficult. Despite advances in the oncological management of disseminated colorectal cancer, surgical resection of liver metastases remains the only therapeutic intervention that offers the possibility of long term survival and cure. A decision for surgical hepatic resection is dependent on both patient and tumour characteristics. For many, it is not a viable option.

The addition of newer chemotherapeutic agents to the established 5-fluorouracil regime has resulted in limited improvement in clinical outcome (Kelly C and Cassidy J, 2007). Clinical trials have shown improved survival in patients with metastatic colorectal cancer when anti angiogenic therapies are combined with conventional chemotherapy (Hurwitz *et al.*, 2004). This novel treatment modality has shown that for optimal oncological management of disseminated colorectal cancer, multiple therapies should be deployed which target different stages of metastasis development. For oncological advances to occur, accurate *in vivo* models are required to study colorectal cancer metastasis development. These models, by increasing our understanding of the early stages of colorectal liver establishment (Fig 1.1.), will facilitate the development of novel therapeutic interventions and allow the clinical effects of these interventions to be studied.

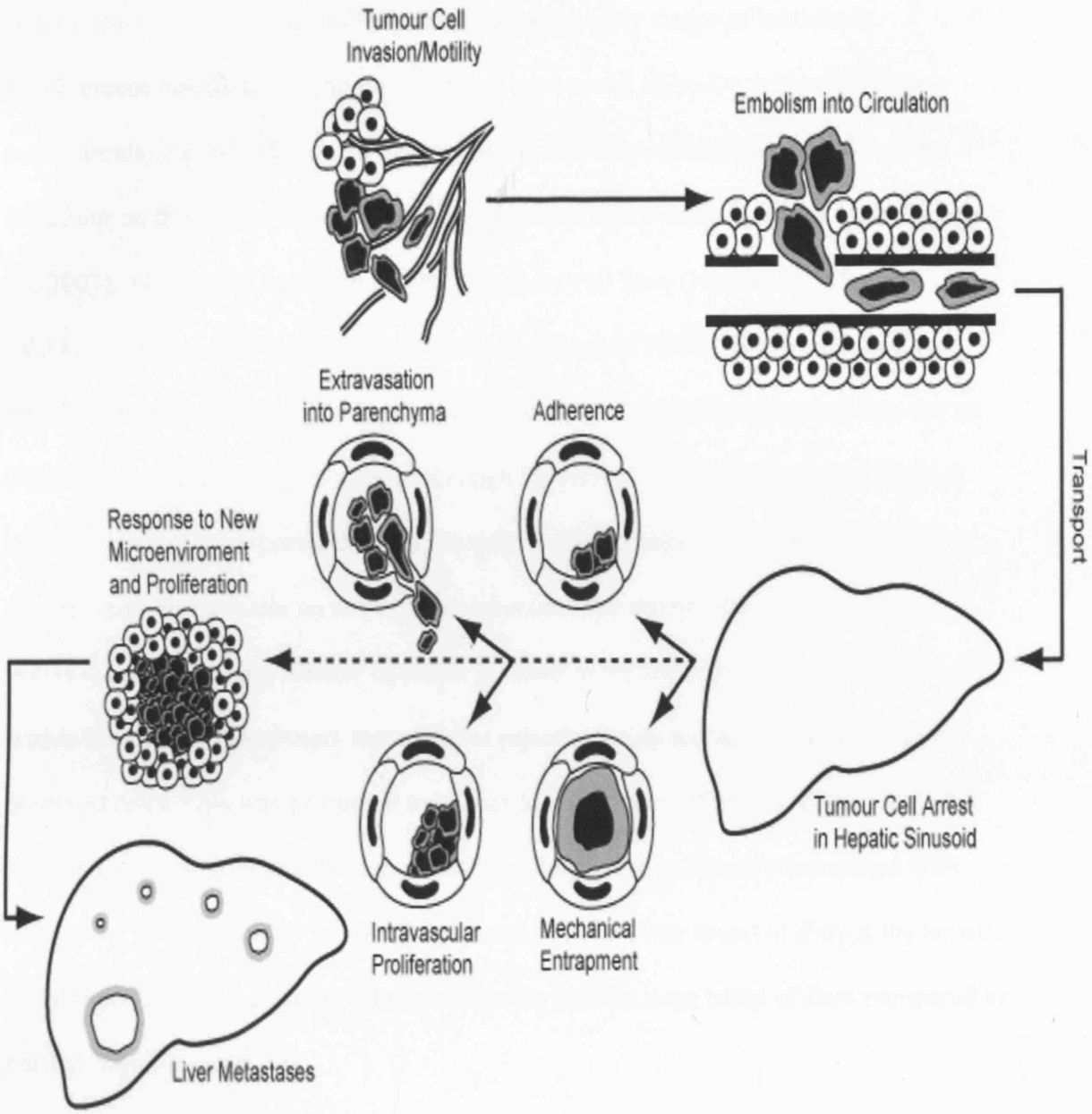


Figure 1 The Development of Colorectal Liver Metastasis.
This figure graphically depicts the metastatic process – focussing on the early stages. Initially the colorectal cancer cells at the primary locus proliferate. Subsequently, these malignant cells become invasive and adopt a migratory phenotype. The invasion process if successful will allow the tumour cells to enter the circulation. In vivo, colorectal cancer cells can metastasise using a haematogenous or lymphatic method. For simplicity, only haematogenous spread is discussed. The haematogenous route of spread is utilised in many recent in vivo studies shown in table 1. The cancer cells are then carried via the portal circulation to the liver where the sinusoids are the vessels with the smallest diameter. The figure then depicts the two different methods proposed for tumour cell arrest – mechanical entrapment and specific tumour cell adhesion. After the initial arrest, there is also evidence to support different methods of early metastatic development. Tumour cells can either undergo extravasation into the parenchyma and subsequent proliferation or undergo initial proliferation intravascularly followed by liver infiltration. If the tumour cells successfully complete these stages, they proliferate within their new environment and can develop into macroscopic liver metastasis.

The creation of a truly biologically accurate model of metastasis has been challenging (see Chapter 3). This thesis will concentrate on the early stages of metastasis development mainly focussing on colorectal cancer cell adhesion within the liver microcirculation and subsequent extravasation. The most established *in vivo* model focussing on this period of metastasis development was created by Haier et al (Haier *et al.*, 2003). This model used two colorectal cancer cell lines (Human HT-29 & rat CC531 colorectal cancer cells). $1x10^6$ cells in one ml of PBS buffer were introduced into the circulation of Sprague Dawley rats. Three methods of introduction into the rat circulation were used – intra-arterial through the carotid artery, intra-venous through jugular vein and intra-portal through the extra-hepatic portal vein. Throughout the experiments, the animals remained haemodynamically stable. Haier et al (Haier *et al.*, 2003) showed that intra arterial injection resulted in the highest number of visible cells within the hepatic circulation. Intra venous injections resulted in significantly fewer observed cells. This was presumed to be due to entrapment of tumour cells within the pulmonary microcirculation. Intravenous injections were commonly associated with pulmonary and circulatory failure. Intra portal injection was found to disrupt the hepatic circulation. This was postulated to be due to the sudden large bolus of flow compared to normal physiological flow.

The colorectal cancer cells were labelled with Calcein AM – a dye that requires intracellular activation and therefore labels only viable cells. The rat liver, which had been exteriorised through laparotomy, was viewed through an intra-vital microscope. In a defined area, labelled colorectal cancer cells were visualised at five minute intervals over half an hour. The total number of cells seen, the number of adherent cells, the number of extravasated cell and the migration rate [defined as the number of extravasated cell/ total number of cells expressed as a percentage] were calculated. The

interactions of the human and rat colorectal cancer cell lines were statistically analysed and no significant differences found.

Haier's model (Haier *et al.*, 2003) has been used to provide many insights into the early development of colorectal liver metastases (Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006). However it is not perfect. Recent experiments (Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006) have used the HT 29 human colorectal cancer cell line in a rodent host. The "seed and soil hypothesis" of metastasis development states that a tumour is a heterogeneous population of cells; selection occurs during the metastasis establishment and that tumour and micro environmental interactions occur between the tumour cell and the host organ (Fidler, 2002;Fidler, 2003). The cancer cell line selected is crucial in maintaining the biological accuracy of the *in vivo* model. The biological accuracy of a cancer model which uses human colorectal cancer cells to develop metastasis in a rat liver has to be questioned. The comparison between human HT29 colorectal cells and rat CC531 colorectal cancer cells showed no differences over the thirty minute observation period (Haier *et al.*, 2003). However the differences between the hepatic architecture and homeostatic mechanisms in a rat and a human are likely to produce a distorted image of metastatic development. Syngeneic models therefore are likely to provide a more accurate representation of metastatic development.

Enns et al has identified integrins as having a crucial role in the early stages of colorectal cancer liver metastasis development (Enns *et al.*, 2004;Enns *et al.*, 2005). There is now much hope that specific inhibitors of these molecules may play a role in cancer therapy (Eble and Haier, 2006). Of particular interest is the finding that integrin subunits $\alpha 2$, $\alpha 6$ and $\beta 4$ are involved in colorectal cancer cell migration. Functional blocking of these integrins inhibits tumour cell extravasation (Enns *et al.*, 2004).

Although initially questioned by Al-Mehdi et al (Al-Mehdi *et al.*, 2000) and supported by other experimental findings (Ito *et al.*, 2001;Sturm *et al.*, 2003), the most recent research involving colorectal cancer suggests that extravasation follows targeting and adhesion (Haier *et al.*, 2003;Enns *et al.*, 2004;Gassmann *et al.*, 2004;Enns *et al.*, 2005). The observed rapid migration into the liver parenchyma (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) protects the cancer cells from haemodynamic stresses. Previous research has demonstrated that fluid shear stress within the circulation can kill tumour cells rapidly. 70% of B16 murine melanoma cells were killed within one hour by physiological shear stresses (Brooks, 1984). This loss of viability was due to mechanical damage to the cell membranes at least in part through an oxygen metabolite mediated process (Albertsson *et al.*, 1995).

Inhibiton of colorectal cancer cell extravasation, not only exposes the cells to lethal haemodynamic stresses, but would also potentiate the chemotherapy response. Blocking of tumour cell migration would directly prevent cells moving into the parenchyma and establishing metastases. This would allow more aggressive local management of metastasis (radiofrequency ablation and surgery) to be deployed. There is hope that the integrins will provide a method of early intervention in the treatment of colorectal liver metastases.

1.2 Aims

The aims of this study are to evaluate the accuracy of current *in vivo* models of early colorectal liver metastasis development and also to evaluate the role of integrin subunits α2, α6, and β4 in the early stages of colorectal liver development.

To analyse the accuracy of the *in vivo* models of early colorectal liver metastasis development, two different colorectal cancer cell lines (rat and human) will be introduced into two different strains of rat (Sprague Dawley and BD1X). This will allow a comparison and an evaluation of the effect of different colorectal cancer cell line-host combinations on early metastasis development. The rates of tumour cell arrest, adhesion and extravasation in the different experimental models will be analysed to see if significant differences occur.

The evaluation of the role of integrin subunits α2, α6, and β4 in the early stages of colorectal liver development will involve both *in vitro* and *in vivo* experiments. Firstly, the two colorectal cancer cell lines (human HT29 and rat DHDK12) will be analysed using the techniques of Immunohistochemistry and Western Blotting to ascertain if these three integrin subunits are expressed.
Then using the rat DHDK12 cell line in Sprague Dawley rats, the role of the integrin subunits α2, α6, and β4 in extravasation will be analysed over a two hour period. Antibodies will be used to functionally block these three integrin subunits. The effect this intervention has on extravasation and migration rates will then be compared with control groups. Statistical analysis will then be used to identify any significant differences in experimental variables.

CHAPTER 2

A Review of Current *In Vivo* Models for Early Development of Colorectal Liver Metastasis

2.1 Introduction

A European study estimated that 2.9 million cancer cases were diagnosed and over 1.7 million cancer deaths occurred in 2004. The second most prevalent form of cancer diagnosed was colorectal cancer and accounted for 13.2% of all cases. Colorectal cancer was responsible for 203,700 deaths (Boyle and Ferlay, 2005). Only lung cancer was responsible for more cancer deaths. Globally each year, almost one million cases of colorectal cancer are registered and almost half a million deaths are attributed to this disease (Parkin *et al.*, 2001). At diagnosis, as many as 25% of colorectal cancer patients have established liver metastases (Kavolius *et al.*, 1996). The primary locus of the colorectal tumour is frequently managed by either surgery alone or in combination with neoadjuvant and adjuvant oncological therapy (Nelson *et al.*, 2001). Management of the metastatic spread to other organs is a therapeutic challenge. Despite advances in the oncological management of disseminated colorectal cancer (Allegra and Sargent, 2005;Twelves *et al.*, 2005), surgical resection of liver metastases remains the only therapeutic intervention that offers the possibility of long term survival and cure (Mutsaerts *et al.*, 2005). This therapeutic option is dependent on both patient and tumour characteristics. For many, it is not a viable option (Scheele *et al.*, 1995).

Intravital videomicroscopy (IVVM) now allows dynamic, real time imaging of biological events in *in vivo* model systems(Fig 2.1). This technique has proven particularly useful in the study of tumour blood flow and the therapeutic effects of vascular disruptive agents (Iga *et al.*, 2006). IVVM has enabled the early development of colorectal metastasis particularly tumour cell arrest, adhesion and extravasation to be analysed in great detail (Table 2.1). For accurate study of novel therapeutic agents targeted at these early stages, an *in vivo* model must be established which closely resembles the genuine tumour development process.

Fig 2.1a.

This IVVM image shows labelled red blood cells within the hepatic circulation in a rodent host. A hepatic venule is seen.

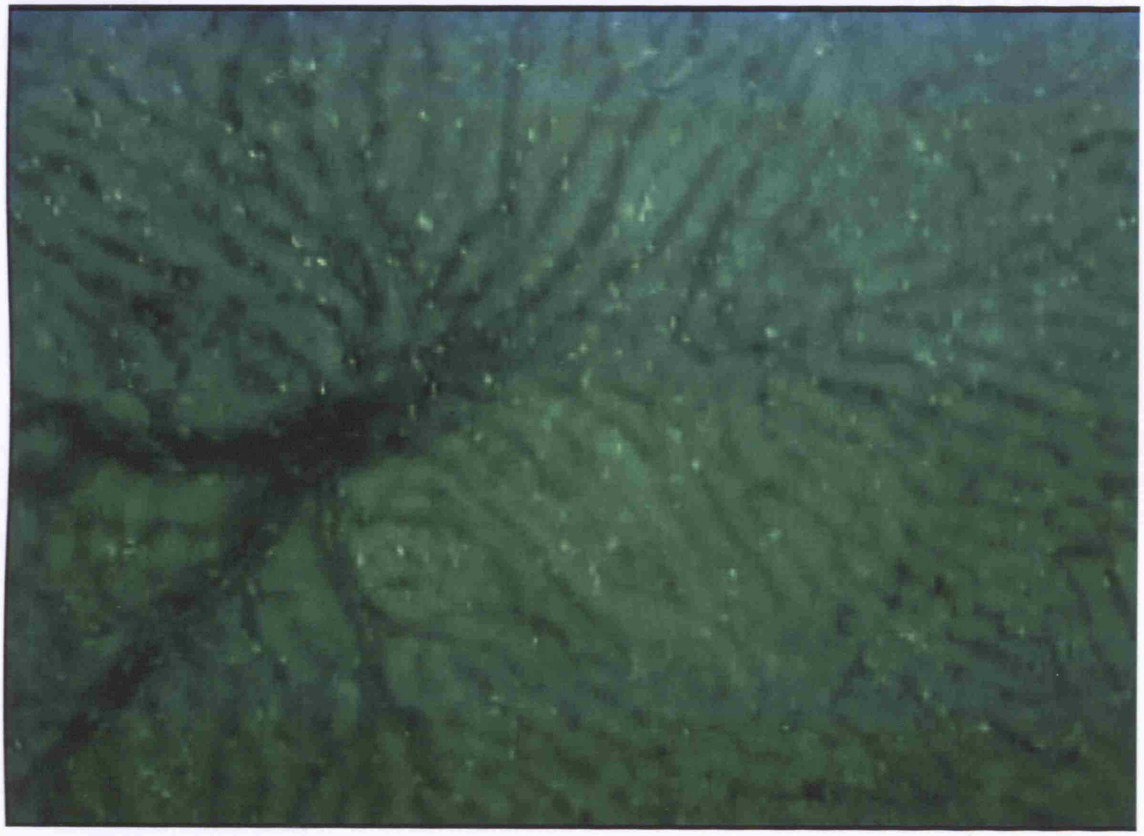
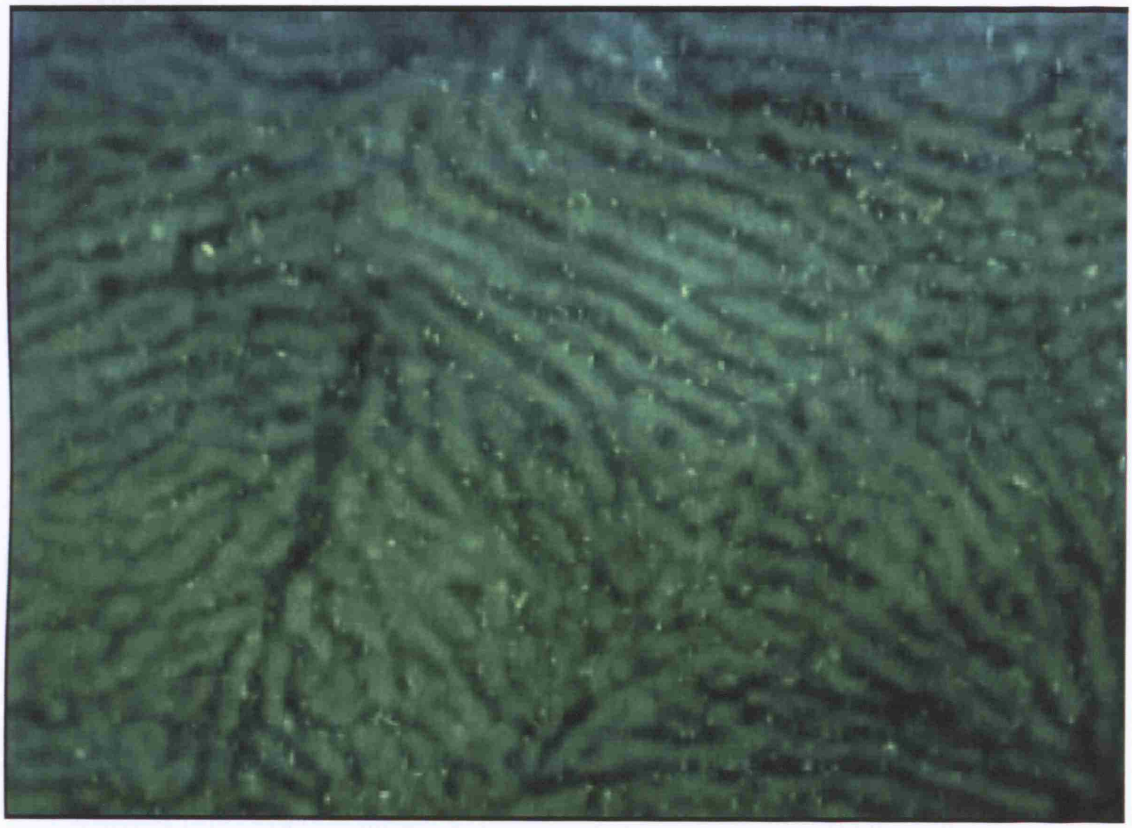


Fig 2.1.b

This image shows labelled red blood cells predominantly within the hepatic sinusoids.



The purpose of this review will be to examine current *in vivo* models of colorectal metastasis. It will analyse the current models focusing on the different techniques used. By providing an overview and analysis of the tumour cell preparation, introduction and monitoring *in vivo*, this review will provide an insight into the problems that can occur. The effects that the different techniques have in altering the picture of metastasis portrayed will also be discussed.

2.2 Model of Metastasis

In vivo models deployed should provide a true reflection of the physiological processes that occur in the cancer patient. As early as 1889, it was proposed that metastasis occurred in a non random pattern. Indeed this was the basis of Stephen Paget's "seed and soil" hypothesis (Paget S., 1889). This hypothesis, as well as the metastasis process, has been studied intensely in the interim period. The "seed and soil" hypothesis has been adapted and now consists of three separate entities (Onn and Fidler, 2002;Fidler, 2003). Firstly, cancers consist of different cell subpopulations each of which has its own phenotypes. Current research (Bao *et al.*, 2006;O'Brien *et al.*, 2007) is closely examining these cell subpopulations to determine whether every cancer cell possesses the ability to initiate and sustain tumour growth or whether only a subset of cells, cancer stem cells, possess such potential. O'Brien et al (O'Brien *et al.*, 2007) identified human colon cancer-initiating cells in immunodeficient mice hosts post transplantation. All human colon cancer-initiating cells were CD133+. The majority of the tumour cells were CD133- and were unable to initiate tumour growth. Bao et al (Bao *et al.*, 2006),in glioma tumours, also identified CD133+ cells. Within glioma tumours *in vitro* and *in vivo*, a higher proportion of these CD133+ cells were found to survive ionising radiation. These CD133+ cells were found to initiate DNA repair, post radiotherapy, more effectively than CD133- cells. These results suggested that the CD133+ cells confer radioresistance on glioma tumours and could cause tumour recurrence post radiation therapy. These results suggest that a cancer hierarchy may exist. Only a proportion of tumour cells may be responsible for tumour proliferation. These stem cells may also be responsible for tumour recurrence after conventional oncological management. Significant advances in oncological management may occur through targeted cytotoxic therapies directed at these cancer stem cells. Secondly, the process of metastasis is selective for tumour cells which have the ability to embolise, invade,

adhere, extravasate and establish metastasis in distant organs. Lastly, metastasis requires multiple interactions between the tumour cell and the regulatory mechanisms of the adjacent microenvironment (Liotta and Kohn, 2001;Fidler, 2002).

The modified hypothesis provides a framework to which any novel *in vivo* model should adhere. Deviation from these principles will result in physiological disruption and produce an inaccurate reflection of metastasis development. Not only will a distorted image be obtained, but the model will be invalid for accurate assessment of therapeutic interventions. An *in vivo* model mirroring the genuine metastatic process would allow each stage of metastasis to be studied and be better understood. An accurate model would also facilitate the development of specific therapeutic interventions and enable the clinical effects of such an intervention to be analysed.

2.3 Colorectal Cancer Cell Line Selection

Perhaps the most significant factor affecting the biological accuracy of the in vivo model is the selection of an appropriate colorectal cancer cell line. Some studies have utilised a syngeneic colorectal cancer cell line (Sturm *et al.*, 2003;Reinmuth *et al.*, 2003;Steinbauer *et al.*, 2003), others have compared syngeneic and non syngeneic lines (Haier *et al.*, 2003),while others have deployed non syngeneic human colorectal cancer cells in a Sprague Dawley host (Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006). Returning to the "seed and soil hypothesis," the third principle states that tumour cells interact with the microenvironment and homeostatic mechanisms (Fidler, 2003). The biological accuracy of a cancer model that uses human colorectal cancer cells to develop metastasis in a rat liver has to be questioned. Although the comparison between human HT29 colorectal cells and rat CC531 colorectal cancer cells showed no differences over the thirty minute observation period (Haier *et al.*, 2003), the differences

between the hepatic architecture and homeostatic mechanisms in a rat and a human are likely to produce a distorted image of metastatic development. Syngeneic models therefore are likely to provide a more accurate representation of metastatic development.

With cultured cell lines, there is the issue of phenotypic drift. Current colorectal cell lines have frequently undergone multiple passages. HT29 human colorectal cancer cells, for example provided by the European Collection of Cell Cultures, have undergone 135 passages. Further culturing of these cells in different laboratories and in different countries is likely to alter tumour cell characteristics. As phenotypic drift occurs, different representations of hepatic metastasis development could be obtained from colorectal cancer cells initially derived from the same cell colony.

2.4 Methods of Transplantation

The "seed and soil hypothesis" states that a tumour is a heterogeneous population of cells; selection occurs during the metastasis establishment and that tumour and microenvironmental interactions occur (Fidler, 2002;Fidler, 2003). Many of the recent models have used colorectal cancer cells cultured *in vitro*. The colorectal cancer cells are labelled with calcein (Haier *et al.*, 2003;Steinbauer *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) or transfected with GFP (Sturm *et al.*, 2003;Steinbauer *et al.*, 2003) prior to introduction to the animal. The tumour cells are then introduced into the circulation of the animal to mimic haematogenous colorectal cancer spread in the human patient (Haier *et al.*, 2003;Steinbauer *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). This model is intrinsically flawed regarding the second principle of the "seed and soil" hypothesis (Fidler, 2003). While the colorectal cancer introduced will be a heterogeneous population and interactions will occur between tumour cells and the

microenvironment, there is no selection occurring. In the human patient, colorectal cancer cells, that reached the blood stream and disseminated, had already exhibited many aggressive or metastatic characteristics. In contrast, colorectal cells that had been passaged *in vitro* have demonstrated none of these characteristics. Current metastatic models could therefore portray an early metastasis development model that is far less efficient than in a human colorectal cancer patient. Genetically engineered animals, that spontaneously develop cancer and subsequent metastases, are the most biologically accurate models of metastasis. Smad3 mutant mice were shown to develop metastatic colorectal cancer (Zhu *et al.*, 1998). Subsequent groups(Philipp-Staheli *et al.*, 2002;Domino *et al.*, 2007) have used these mice to examine different factors influencing colorectal cancer development and progression. However these models are not amenable to the intravital microscope – an essential tool for real time observation of tumour development. Tumour cells must be labelled to allow detection in vivo.

Direct haematogenous introduction negates the effect of immune surveillance. Physiological immune surveillance mechanisms are artificially challenged by haematogenous bolus introduction of tumour cells to varying extents as the immune system would take time to detect and process the newly introduced cellular matter. The variability of the immune response between species and individuals within species may account for experimental differences seen in tumour natural history. Recent research using orthotopic tumour models examined human colorectal cancer in female BALB/c mice (Flatmark *et al.*, 2004). 12 colorectal cancer strains were implanted. The results showed considerable variation between the cell lines with regard to tumour propagation and dissemination. One of the twelve colorectal cancer cell lines produced liver metastasis and this only occurred in two of ten animals. While the method of colorectal cancer cell introduction is different – orthotopic versus haematogenous –

immunosurveillance and clearance was occurring. This theory is further supported by other orthotopic research. A further study looking at HT29 human colorectal cancer cells produced similar results (Flatmark *et al.*, 2004) in immunocompetent mice (Guilbaud *et al.*, 2001). However in the immunocompromised SCID mouse, significantly higher rates of lymph-node metastases and liver and lung metastases occurred. Immune reactions have also been observed in longer haematogenous experimental models. CT 26 syngeneic murine colorectal cancer cells were introduced into a BALB/c mouse host (Steinbauer *et al.*, 2003). Metastatic development was monitored and comparisons made between cells labelled with GFP and cells labelled with conventional techniques. No significant differences were noted in early metastasis development. However, a significant reduction in the late metastatic growth of GFP expressing CT26 colorectal cancer cells was observed. This phenomenon was only noticed in the immunocompetent mice. Further experiments in immunodeficient mice demonstrated that this cancer cell clearance was immune mediated. In the previously healthy colorectal cancer patient, colorectal cancer development and dissemination will occur despite immunosurveillance in an immunocompetent patient. A sudden introduction of $1x10^6$ colorectal cancer cells (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) into the circulation will not accurately portray tumour cell dissemination. Instead the speed of introduction will allow at least initial escape from the immune system. This may in part explain why when human HT29 colorectal cells and rat CC531 colorectal cancer cells were introduced haematogenously into Sprague Dawley rats (Haier *et al.*, 2003), no significant differences were found between the adhesive properties of the two cell lines over the 30 minute observation period. The introduction of a cell bolus and the short duration of the experiment will prevent immune surveillance from influencing the early stages of hepatic metastasis development. In longer experiments, significant differences are likely to be detected between the growth

of these two tumour cell lines. In the rodent host, the human cancer cell line is more likely to be recognised as foreign matter and an immune response triggered. This response is likely to inhibit metastasis development.

Arguments continue whether haematogenous or orthotopic introduction of colorectal cancer cells provide a more accurate model. Orthotopic implantation (Guilbaud *et al.*, 2001;Sturm *et al.*, 2003;Reinmuth *et al.*, 2003;Flatmark *et al.*, 2004) from the principles of the "seed and soil hypothesis" is likely to portray a more accurate picture of the metastasis process. A heterogeneous population of colorectal cancer cells is allowed to establish itself. Selection will occur throughout the dissemination process and tumour microenvironment interactions occur. The disadvantage is that this process of implantation makes analysis of events in early metastasis development hard to analyse with any degree of accuracy. Both theories of mechanical entrapment (Naumov *et al.*, 1999;Chambers *et al.*, 2001;MacDonald *et al.*, 2002) and cell specific adhesion (Sturm *et al.*, 2003;Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) have been proposed for colorectal cell arrest within the hepatic microcirculation. A model of intravascular proliferation of tumour cells prior to extravasation has also been described (Al-Mehdi *et al.*, 2000;Sturm *et al.*, 2003). Accurate analysis of the above steps requires large numbers of tumour cells within the circulation at any specific time point. The bolus of cells, introduced in the haematogenous in vivo models, makes analysis of the early steps of metastasis development much easier. With the orthotopic model, the number of tumour cells within the circulation at any given time point will be significantly less than the haematogenous bolus. In addition in the orthotopic model, the introduction of tumour cells into circulation will be more erratic. Observations and experiments will be prolonged. This not only will increase observation error and variation, thereby reducing

statistical validity but present problems with preservation of normal physiological variables in the host.

In the haematogenous models, calein AM is frequently used to label the colorectal cancer cells (Sturm *et al.*, 2003;Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). The principle focus of these experiments is to study the very early stages of metastasis development – tumour cell arrest, adhesion and extravasation. These experiments are short – minutes to hours. The calcein AM is not only an effective cellular label for this purpose but is not known to have any physiological effect on the tumour cell. The study of tumour cell arrest, adhesion and extravasation in orthotopic models is more difficult from a tumour cell labelling perspective. Orthotopic experiments are prolonged and involve colorectal cancer cell division. This cell division creates problems with calcein AM and nanosphere fluorescent probes (MacDonald *et al.*, 2002) as will be discussed in the next section. In order to optimise tumour cell signal, either GFP or exogenous administration of polymer substrate would have to be used. GFP has been shown to stimulate an immune reaction in longer experiments (Steinbauer *et al.*, 2003). Early metastasis development in the liver, after colorectal cancer cell dissemination from the primary site of orthotopic implantation, is likely to be affected . The alternative labelling technique involves the haematogenous administration of polymer substrate (Weissleder *et al.*, 1999). Haematogenous polymer could influence the early stages of metastasis development. This technique (Weissleder *et al.*, 1999) is also more appropriate for labelling metastases than individual cells and is associated with high background liver fluorescence(Hoffman, 2002).

2.5 Host and Surgical Techniques Used

To date, most *in vivo* models studying the early stages of colorectal liver development have used rodents (Table 2.1). For metastasis development to occur, multiple interactions occur between the introduced tumour cells and rodent liver. To ensure an accurate clinical picture, a cell line comparable with these hosts should be selected. This will be discussed in more detail later. At best, these early models will provide only some representation of the human metastasis process as there is no close correlation between the species. Models in higher primates are therefore likely to provide more accurate representation. However, higher order primate studies are yet to emerge.

The host and particularly techniques deployed to establish the experimental model can affect the validity of the metastasis model. Much current work relies upon the host, under anaesthetic, undergoing an abdominal incision (Sturm *et al.*, 2003;Haier *et al.*, 2003;Reinmuth *et al.*, 2003;Steinbauer *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). The anaesthetic and the stress of surgery introduce novel variables into the metastasis model. In addition, subsequent exposition of the liver, involving mobilisation and partial exteriorisation for IVVM observation, can distort metastasis development (Sturm *et al.*, 2003;Haier *et al.*, 2003;Steinbauer *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). This mobilisation of the liver has the ability to disrupt the hepatic architecture and microenvironment of the colorectal cells. Vessels in the microcirculation could easily become compressed. It has been argued by some (Enns *et al.*, 2005) that this compression could be in part responsible for tumour cell mechanical entrapment (Koop *et al.*, 1995;Naumov *et al.*, 1999). Trauma sustained during mobilisation could also trigger inflammatory responses which would add yet another variable to the metastasis model.

Table 2.1. Evolution of *in vivo* studies examining the early stages of colorectal cancer liver metastasis using murine models.

Study	Strain Of Animal	Cancer Cell Line	Molecular Target	Labelling Method	Tumour Inoculation	Outcomes
(Luzzi *et al.*, 1998)	C57BL/6 mice	B16F1 murine melanoma	NONE	Fluoresbrite carboxylated polystyrene nanospheres	Superior Mesenteric Vein	Adhesion and extravasation process is very efficient (>80% survive). The inefficiency of metastatic process occurs after this early stage.
(Naumov *et al.*, 1999)	SCID mice	CHO-K1	NONE	GFP	Mesenteric Vein	GFP expressing cells enables the metastatic process to be monitored *in vivo*
(Ito *et al.*, 2001)	athymic nude mice of KSN strain	Rat tongue carcinoma RSC3 LM & E2	NONE	GFP	Intra portal via mesenteric vein	Metastatic cells and non metastatic cells arrest in hepatic circulation. After 3 days all non metastatic cells cleared from liver. Metastatic cells from stable attachment.
(Ding *et al.*, 2001)	Balb/c mice – P selectin knockout mice and wild type C57BL/6	C26 adeno-carcinoma & EL-4 lymphoma	Selectins (function blocking mAb)	PKH-26	Mesenteric Vein	Colorectal cancer cells demonstrated mechanical entrapment. Lymphoma cells metastasised although these cells were smaller than the sinusoid diameter. Cell specific adhesions using P selectin are involved.
(Kikkawa *et al.*, 2002)	Balb/c mice	CHO-K1	αvβ3 (cells transfected with αvβ3)	GFP	Portal vein and tail vein	Transfected αvβ3 showed significantly higher accumulation in the liver post portal vein injection but not in the lung after tail vein injection compared to wild type. αvβ3 appeared to promote extravasation.
(Reinmuth *et al.*, 2003)	Balb/c mice	Murine CT26 adeno-carcinoma	αvβ3 & αvβ5 (S247 & αvβ3/ αvβ5 antagonist)	None	Spleen	S247 prolonged survival in this animal model. S247 impaired both metastatic and angiogenic processes.
(Steinbauer *et al.*, 2003)	Balb/c mice – SCID and wild type	Murine CT26 adeno-carcinoma	NONE	GFP and calcein AM	Portal vein	GFP stain in longer experiments can trigger an immune reaction.
(Sturm *et al.*, 2003)	Balb/c mice	Murine CT26 adeno-carcinoma	NONE	GFP	Spleen	Created an animal model for colorectal cancer using murine cancer cells in a murine host.

Table 2.2. Evolution of *in vivo* studies examining the early stages of colorectal cancer liver metastasis using rat models.

Study	Strain Of Animal	Cancer Cell Line	Molecular Target	Labelling Method	Tumour Inoculation	Outcomes
(Haier *et al.*, 2003)	Sprague- Dawley rats	Human HT-29 & rat CC531 colorectal cancer cells	NONE (none)	Calcein AM	Intra arterially, intra venous and extrahepatic portal vein	Created animal model without GFP label, which could cause immune reaction. Showed despite method of colorectal cancer cell inoculation, cells still metastasised to liver.
(Enns *et al.*, 2004)	Sprague-Dawley rats	Human HT-29 cells (HT-29P & HT-29LMM)	Integrins – α1, α3, α5, α6, β4 and α2β1. VCAM-1 and Selectins	Calcein AM	Intra cardiac	Specific integrins play a key role in colorectal cancer cell adhesion to the liver and in tumour migration. ECM of space of Disse is important in metastasis formation.
(Enns *et al.*, 2005)	Sprague- Dawley rats	Human HT-29 colorectal cancer cells	Intergins pan αv, αvβ3 and αvβ5	Calcein AM	Intra arterial	αv integrins esp αvβ5 have a key role in colorectal cancer cell adhesion to the liver.
(Schluter *et al.*, 2006)	Sprague-Dawley or nude rats	HT 29P low, KM-12C Intermediate or HT-29LMM, KM-12L4 High metastatic colorectal cancer cells		Calcein AM	Intra-arterial	Cell adhesion occurred in metastatic target organs only. Migration into target organs correlated with their metastatic potential.

Cannulation of the blood vessels required for colorectal cancer cell introduction has recognised complications and effects on the animal host and the introduced cell line (Kikkawa *et al.*, 2002;Haier *et al.*, 2003;Steinbauer *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). These problems and resulting distortion to the metastasis development are likely to be more significant in smaller rodents. In mice, the tissues are more friable and mobilisation of the liver can induce more significant trauma. During an experiment, alterations to the normal physiological variables of the animal host can occur which again will be more exaggerated in a smaller host. In addition to the stresses of anaesthetic and laparotomy, further stresses from insensible loss and hypovolaemia and hypothermia can occur during the experiment. Even with close monitoring of pulse, mean arterial pressure, pulse oximetry and temperature and adherence to protocols to maintain values within normal variables, this is an artificial system at best. Another host factor to consider is the immune status. It has been shown in longer experiments that the immune system can play a critical role (Guilbaud *et al.*, 2001;Steinbauer *et al.*, 2003;Flatmark *et al.*, 2004). SCID animal hosts used to accommodate xenogenic cell lines greatly alter the normal physiological criteria (Guilbaud *et al.*, 2001).

The method of tumour cell introduction can have a significant effect on metastasis development. Earlier models used intra-portal routes via mesenteric veins (Luzzi *et al.*, 1998;Naumov *et al.*, 1999;Ding *et al.*, 2001;Ito *et al.*, 2001;Kikkawa *et al.*, 2002;Steinbauer *et al.*, 2003). This method of introduction not only caused transient disruption of a usually low pressure system but could account for the phenomenon of mechanical entrapment (Ding *et al.*, 2001;Ito *et al.*, 2001). Mechanical entrapment is a theory that proposes that tumour cell arrest occurs when larger tumour cells become lodged in microcirculation vessels. Many argue this is artefactual due to either

compression of the hepatic architecture or circulatory disruption. Regardless of whether tumour cell arrest is due to mechanical entrapment or cell specific adhesion, these models provide useful information about cancer cell extravasation and cancer cell growth in a foreign environment (Luzzi *et al.*, 1998;Naumov *et al.*, 1999;Ding *et al.*, 2001;Ito *et al.*, 2001;Kikkawa *et al.*, 2002;Steinbauer *et al.*, 2003). However, clarification of the mechanism of tumour cell arrest is extremely important. If tumour cells become trapped in the microcirculation due to size restriction – mechanical entrapment, creation of a therapeutic intervention to inhibit this is futile. If, as many argue (Ding *et al.*, 2001;Ito *et al.*, 2001), cell specific adhesion is responsible then therapeutic interventions targeting the responsible molecules could significantly impair metastasis development. The argument, that mechanical entrapement is artefactual, is strongly supported by the findings of Haier et al (Haier *et al.*, 2003). Their *in vivo* model introduced $1x10^6$cells in 1ml of PBS solution. Venous tumour cell introduction via the jugular vein could cause cardio-pulmonary dysfunction. Intra-portal introduction was noted, due to transient dramatic pressure changes, to cause hepatic physiological disruption. The preferred method of introduction was arterial via the carotid artery. Physiological disruption was minimalized and the number of adherent cells viewed in the hepatic circulation by IVVM was greater than with other methods. Through a series of experiments (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006), this group has shown that colorectal cancer cells adhere only in metastatic target organs and this is shown to occur in vessels with diameters larger than the colorectal cancer cells. Adaptations to the original model (Haier *et al.*, 2003) have shown that blocking specific integrins can significantly reduce colorectal cancer cell adhesion (Enns *et al.*, 2004;Enns *et al.*, 2005) and migration (Enns *et al.*, 2004). These models clearly demonstrate specific colorectal organ targeting mediated by cell surface receptors. Most recently, comparison of colorectal cancer cell lines of varying

metastatic potential has shown that migration rates correlate with metastatic potential (Schluter *et al.*, 2006).

Reinmuth et al (Reinmuth *et al.*, 2003) created an *in vivo* model using a murine host and a murine colorectal cancer cell line. The tumour cells were introduced intra-splenically to induce metastases. Administration of S247, a peptide inhibitor of integrin αvβ3, was shown to prolong survival, decrease colorectal metastasis and angiogenesis. Models like these help elucidate the essential processes and molecules involved in metastasis establishment and development. Although identified in a distantly related species, they provide the insights that are essential for increased understanding of the metastasis process and the development of highly specific cancer targeted therapy.

2.6 Cell Labelling Methods

To enable the colorectal cancer cells to be viewed under the intravital microscope, the cells must be suitably labelled. Traditional labelling methods have utilised various histochemical marker genes -Escherichia Coli β-galactosidase gene (Lac Z), Drosophila alcohol dehydrogenase gene (ADH) and human placenta alkaline phosphatase gene (ALP). These have been cloned into eukaryotic cells using viral vectors. The cells have subsequently been differentiated using appropriate staining protocols (Lin *et al.*, 1990;Lin and Culp, 1991). Although good for analysing cells in culture systems, these markers require cellular staining and therefore the sacrifice of the animal. These techniques are ineffective for dynamic *in vivo* assessment of cancer progression.

Adaptations to conventional markers have occurred. In particular modifications of the Lac Z labelling system has allowed *in vivo* real time detection of β-galactosidase activity (Tung *et al.*, 2004). These adaptations utilise complex synthetic graft polymers.

When cleaved by target proteases, the proteins become fluorescent in the infra-red region of the spectrum (Weissleder *et al.*, 1999;Tung *et al.*, 2000;Chen *et al.*, 2002). The polymer substrate can be adapted to the specific protease. Since many proteases are cellular specific, specific cancer cells can be monitored. While specifically labelling target cells in the animal, there are some limitations to this system. This system relies on an intravenous infusion (Figueiredo *et al.*, 2006) of infra-red probes into the animal host which entails non selective delivery to the animal organs. Due to the abundance of proteases within hepatocytes, concerns have been expressed (Hoffman, 2002) about high liver background fluorescence. The study of metastasis development involves monitoring single labelled cancer cells undergoing tumour cell arrest, adhesion and extravasation (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). The high background fluorescence of the liver will impede accurate analysis of liver metastasis development. Experimental models investigating the early stages of colorectal metastasis development frequently use a haematogenous route of tumour cell dispersion to mimic blood borne tumour spread. The delivery of infra-red probes by intravenous infusion is likely to distort the physiological picture seen in the experimental model. Not only the volume of the infusion, but the introduction of foreign material into the blood stream simultaneously to the colorectal tumour cells, is likely to provide an inaccurate picture of the metastasis process. Recent studies (Enns *et al.*, 2004;Enns *et al.*, 2005) have shown adherence of tumour cells within the hepatic microcirculation to occur within minutes of injection. Delivery of a substrate for enzymatic cleavage would have a delay before tumour cells fluoresced due to substrate delivery to cells and cellular processing of the substrate. This makes the method unsuitable for the very early stages of metastasis development. Another method of tumour cell labelling uses the luciferase gene which involved exogenous delivery of substrate (Sweeney *et al.*, 1999). This marker gene has been transfected into human cancer cells to monitor tumour

growth and regression. Disruption to normal physiology especially during the early stages of tumour dissemination definitely occurs with this system. The luciferase enzyme requires the substrate luciferin to be delivered for light emission. In addition, due to low image resolution and signal achieved, an anaesthetic is required. Both these systems of exogenous substrate delivery are therefore better suited to the monitoring and identification of established tumour cells in the *in vivo* animal model. Antibodies have been used in cancer imaging but again are more suitable for imaging established tumour metastasis (Chester *et al.*, 2004).

Fluorescent labelling of the colorectal cancers prior to the introduction into the animal host is considered favourable for monitoring the early stages of dissemination. This was achieved either by tumour cell transfection and altered protein expression (Hoffman, 2002) or introducing fluorescent compounds into the cell (MacDonald *et al.*, 2002). These methods allow immediate visualisation of haematogenously introduced colorectal cancer cells. Green fluorescent protein (GFP) cDNA extracted from A.Victoria has been transfected into both pro and eukaryotic cells with stable expression of protein obtained (Chalfie *et al.*, 1994). Optimisation of GFP expression and signal strength has subsequently been achieved (Cheng *et al.*, 1996;Zolotukhin *et al.*, 1996;Cormack *et al.*, 1996). This has enabled GFP to be used extensively as a tool for cancer research. Hoffman (Hoffman, 2002) has written an excellent review on GFP and its uses. Recently, several groups utilised GFP to study the early stages of colorectal cancer spread (Sturm *et al.*, 2003;Steinbauer *et al.*, 2003). This protein has many advantages compared to other methods of colorectal cancer cell labelling. In cells lines with stable expression, GFP continues to be expressed in future generations (Hoffman, 2002). This enables easy observation of tumour growth over a prolonged period of time. Comparatively, calcein AM (Uggeri *et al.*, 2004) and other non-toxic cytoplasmic

markers produce fluorescent signals that tend to be relatively short lived and susceptible to bleaching with exposure to fluorescent illumination (MacDonald *et al.*, 2002). Fluorescent nanospheres have been deployed in cancer cell labelling. These markers tend to be photoresistant. However the distribution of these spheres throughout the cell can be uneven and during replication the number of spheres per cell diminishes, leading to decreased signal strength (MacDonald *et al.*, 2002). Introduction of nanospheres into cancer cells is likely to alter the characteristics of the tumour cell. GFP appeared the perfect tumour cell marker. Recently problems have been identified with this marker. GFP has now been shown to stimulate an immune reaction in longer experiments. This was noticed in murine colorectal cancer introduced into a murine host (Steinbauer *et al.*, 2003).

As yet, the ideal colorectal cancer cell label for *in vivo* staining has not been discovered. Tumour cell labelling prior to introduction appears to be the most favoured and sensible approach and is utilised in recent experimental systems. Table 1 shows the experimental model development and the refinement of the model to analyse the early stages of colorectal metastasis development. Recent works have used Calcein AM (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). Despite its disadvantages, calcein AM has not been shown to cause any immune reaction nor to have an effect on key tumour cell functions. In HT29 human colorectal cancer, calcein AM has been shown to have no effect on tumour cell viability or adhesion (Haier *et al.*, 1999a). In addition, as calcein AM is activated intra-cellularly, it will only label viable tumour cells (Haier *et al.*, 2003;Uggeri *et al.*, 2004). It therefore fulfils the role of an efficient tumour cell label as it causes minimal disruption of normal physiological processes.

2.7 Intravital Microscope

Another factor which can influence the experimental model and metastasis development is the type of intravital microscope used (Fig 2). The two types of intravital microscope are upright (Haier *et al.*, 2003;Steinbauer *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) and inverted (Naumov *et al.*, 1999;MacDonald *et al.*, 2002;Sturm *et al.*, 2003). Both of these have effects on part of the third principle of the "seed and soil" hypothesis – the tumour microenvironment. In the upright scope, the liver is mobilised, exteriorised and viewed in an animal host lying on its back. This process involves dissection of the falciform ligament, mobilising and stretching some of the vasculature. The liver is placed on a viewing platform outside and above the host. This process can distort the hepatic microenvironment. Exteriorisation of the liver involves more extensive physical handling and experimental exposure of the liver to the external environment than is associated with the inverted IVVM. All of these factors could have profound effects on the circulation, causing either vasoconstriction or vasodilatation.

The inverted microscope involves surgical exposition of the liver with minimal mobilisation. The animal is placed on its abdomen with the liver above the viewing platform. Using the inverted microscope, the liver is likely to have less physical manipulation but is likely to have more compression. This can distort the architecture of the liver, especially the microcirculation. The microcirculation, particularly the sinusoids (Naumov *et al.*, 1999;Sturm *et al.*, 2003;Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005), appear to play an important role in tumour cell arrest. This tumour cell arrest is vitally important. The mechanism of how this arrest occurs is still contested. Cell specific adhesions (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) accounting for tumour cell arrest would allow targeted interventions to be created. Mechanical entrapment (Naumov *et al.*, 1999;Chambers *et al.*, 2002), thought

by some to be an artefact associated with compression of the hepatic microcirculation (Enns *et al.*, 2004), would allow very little therapeutic intervention.

2.8 Discussion

As yet, the perfect model of early colorectal cancer dissemination and metastasis development has not been established. For close analysis of early events, the haematogenous method is preferred over orthotopic as it allows studying of larger numbers of tumour cells to be examined. Calcein AM appears to be an effective mechanism of labelling with minimal distortion of the physiological picture. Syngenic cell lines are likely to provide a more accurate picture of the metastatic process while both methods of intravital microscope visualisation can contribute to the experimental variables. It is only with the establishment of a model that adheres to all of the principles of the "seed and soil" hypothesis that a truly accurate picture of tumour evolution will be seen. An *in vivo* model closely mirroring the genuine metastatic process allows accurate study and an increased understanding of the early stages of colorectal liver metastasis establishment and development. Such a model would facilitate the development of specific therapeutic interventions and enables the biological effects of these interventions to be analysed (Eble and Haier, 2006).

CHAPTER 3

A Review of the role of integrins in the early stages of colorectal liver metastasis and their potential as a therapeutic target

3.1 Introduction

Recently, the role of cell adhesion molecules in liver metastasis development and their potential as targets for therapeutic intervention has been examined. Four families of adhesion molecules have been identified - the integrins, the immunoglobulin superfamily, the selectins and the cadherins (Elangbam *et al.*, 1997). The integrin family (Hynes, 1987) are known to play a key role in many crucial stages of metastasis. Indeed, antagonists of specific integrins have been evaluated as novel anti cancer agents (Kerr *et al.*, 2002). Vitaxin(Posey *et al.*, 2001;Patel *et al.*, 2001) and cilengitide(Eskens *et al.*, 2003;Smith, 2003) are being tested as future anti cancer treatments. S247 (Reinmuth *et al.*, 2003), an αvβ3 antagonist, has been shown both to decrease colon cancer liver metastases and angiogenesis in mice and to improve survival. Inhibition of α5β1 function with a small peptide (ATN161) in combination with flurouracil infusion has reduced colorectal liver metastases and improved survival in mice (Stoeltzing *et al.*, 2003).

Although the integrins have a role in tumour development at the primary locus and throughout the metastatic process (Eble and Haier, 2006;Janes and Watt, 2006;White *et al.*, 2006;Wolf and Friedl, 2006;Tucker, 2006), this review focuses specifically on integrin involvement in the early stages of colorectal liver metastasis especially in selective tumour cell adhesion and extravasation. It examines the current knowledge of integrin participation in this area and highlights the future therapeutic implications.

3.2 Integrins

Integrins are a group of type 1 transmembrane glycoproteins found only in metazoans or multi cellular animals. Since their recognition by Hynes in 1987 (Hynes, 1987), the integrin family has been studied intently. In more advanced animals, increased numbers of integrins are found to accommodate complex developmental processes (Hynes, 2002). Integrins exist as heterodimers with two constituent subunits α and β (Calderwood, 2004). In humans, 18 α subunits and 8 β are known and these interact to form at least 24 different heterodimers. The integrins have large extracellular domains and with the exception of β4 (Hynes, 2002), they have short cytoplasmic tails. The function of the large extracellular domains is to bind ligands, with different heterodimers having different ligand selectivity(Bokel and Brown, 2002) (Table 3.1).

Table 3.1 Shows different integrin ligand selectivity(Jin and Varner, 2004)

Integrin	**Ligand Selectivity**
	β1 Family
α1β1	Laminin collagen
α2β1	Laminin collagen α3β1
α3β1	Laminin collagen fibronectin epiligrin entactin α2β1
α4β1	Fibronectin (CS-1) VCAM
α5β1	Fibronectin (RGD) Li-CAM Fibrinogen
α6β1	Laminin merosin kalinin
α7β1	Laminin
α8β1	Fibronectin
α9β1	Tenascin
αvβ1	Fibronectin Vitronectin
	β2 Family
αLβ2	ICAM-1 ICAM-2 ICAM-3
αMβ2	iC3b fibrinogen Factor X, ICAM-1
αXβ2	iC3b fibrinogen
	Platelet Integrin
αIIbβ3	Fibrinogen Fibronectin von Willebrand's Factor
	αv Family
αvβ3	Vitronectin fibrinogen von willebrand's factor denatured collagen, thrombospondin, Del1, Cyr 61 FISP
αvβ1	Fibronectin vitronectin
αvβ5	Fibronectin osteopontin Del1
αvβ6	Fibronectin
αvβ8	Fibronectin
	Other Known Combinations
α6β4	Laminin
α4β7	VCAM, MADCAM Fibronectin (CS-1)

The crystal structure of the extracellular domain of the αvβ3 integrin in isolation and binding to an Arg-Gly-Asp peptide sequence has recently been elicited (Xiong *et al.*, 2001;Xiong *et al.*, 2002). These molecules have been shown to regulate a diverse range of physiological processes (Hynes, 2002) using two distinct signalling methods - the "outside in" and "inside out" mechanisms (Calderwood, 2004). In the "outside in" mechanism, external ligand binding activates intra-cellular signalling cascades through the cytoplasmic domains. When prostate cancer binds to the appropriate microenvironment, intracellular calcium levels are shown to be increased, a change that frequently stimulates cell proliferation. This was inhibited by blocking the αvβ3 integrin and demonstrates the "outside in" signalling mechanism (Lecrone *et al.*, 2000). In the "inside out" mechanism, changes to the cytoplasmic domains alter the structure of the integrin heterodimer and alter the integrin from an inactive to an active form (Emsley *et al.*, 2000;Xiong *et al.*, 2001;Xiong *et al.*, 2002;Shimaoka *et al.*, 2003;Calderwood, 2004). In specific integrins (Felding-Habermann *et al.*, 2001), this alteration increases ligand binding affinity. *In vivo* research, using melanoma cancer cells, has shown the αvβ3 integrin can mediate tumour cell arrest under haemodynamic flow conditions (Pilch *et al.*, 2002). This is dependent on this integrin being in the active state. The "inside out" mechanism allows cells especially tumour cells to target specific organs (Felding-Habermann *et al.*, 2001;Cooper *et al.*, 2002).

These molecules have also been shown to exist in two separate states – active and inactive (Calderwood, 2004). These dynamic molecules play a key role in normal physiological development as has been seen in knock out mutants (Hynes, 2002). It has been proposed that the integrin family are as at least as important to cells as traditional growth factors (Hynes, 2002). Support for this theory comes from research showing

αvβ3 integrin regulates vascular endothelial growth factor VEGF production in certain tumour cells (De *et al.*, 2005).

3.3 The roles of integrins in the early stages of metastasis development

3.3.1 Organ Tropism

The liver is the most common and critical site of distant metastasis of colorectal carcinoma. Colorectal cancer cells metastasize to the liver in a highly specific fashion. The integrins participate to this organ tropism.

Recent work (Schluter *et al.*, 2006) showed that colon carcinoma cells arrest in target organs. This arrest is not due to size restriction. The tumour cells arrest only occurred in metastatic organs. For this to occur and subsequent growth of metastasis in such a specific fashion, specific interactions between the colorectal cancer cell and the organ of metastasis must occur.

Complex interactions occur between the colorectal cancer cells and the liver which are facilitated through the unique hepatic microvascular architecture. Fenestrae are present between the endothelial cells lining the hepatic sinusoid walls and constituting 6-8% of the total surface area (Wisse *et al.*, 1996). The fenestrae expose the extracellular matrix (ECM) components, in the space of Disse, to the circulation (Wisse *et al.*, 1996) and simultaneously allow the blood plasma to percolate through to the hepatocytes. In the space of Disse, pericytes (Wisse *et al.*, 1996) play a major role in the synthesis of the extracellular matrix and produce a variety of collagens, fibronectin and laminin (Baranov *et al.*, 1990;Kitayama *et al.*, 1999). The distribution of these ECM components is varied. Collagen Type I and pro collagen are located at the portal triads and in the space of Disse. Type IV Collagen is detectable in the basement membrane of

the portal structures and intermittently in the space of Disse (Hahn *et al.*, 1980). Fibronectin is abundant in the space of Disse and forms a continuous layer between the hepatocytes and the endothelial cells. Laminin is located both along the sinusoids and abundantly in the cytosol of the portal and hepatic veins (Baranov *et al.*, 1990;Kitayama *et al.*, 1999). Vitronectin is scarce (Martinez-Hernandez and Amenta, 1993). Importantly, this subendothelial layer has an incomplete basement membrane (Martinez-Hernandez and Amenta, 1993;Kemperman *et al.*, 1995). The protruding hepatic ECM-colorectal cancer integrins interactions are proposed to facilitate cell specific targeting and adhesion in colorectal cancer. Haier et al (Haier *et al.*, 2003) in an *in vivo* model showed that despite the method of tumour cell introduction – arterial, venous or portal – metastasis still occurred in the liver. Further work (Enns *et al.*, 2004;Enns *et al.*, 2005) showed that the integrins mediated tumour cell arrest and adhesion. The interactions between the integrins on the colorectal cancer cell surface and the unique hepatic sinusoid architecture contributes significantly to the organ tropism phenomenon.

Further evidence to support the key role of integrins in organ tropism has come from other recent work (Sato *et al.*, 2003). Integrin expression was analysed in both primary colorectal cancer tumours and in resultant pulmonary metastases. In the pulmonary metastases, a significantly higher integrin αv expression was found. There was no difference in DNA ploidy. The integrin expression in the colorectal cancer cells had altered and this is suspected to target the cells to a new but specific organ. This alteration in integrin expression has been seen in other research (Roela *et al.*, 2003). A wild type control group and colorectal cancer cells treated with DMSO to induce growth arrest were compared. Integrin expression and function was compared. Differences were observed between the two groups leading to differences in tumour cell adhesion and

migration. Environmental stimuli can influence colorectal cancer cell characteristics (Brabletz *et al.*, 2004). When colorectal cancer cells bound to Collagen Type 1 through β1 integrin, an intracellular down regulation of Cdx2 was induced – leading to a loss of cellular differentiation.

The integrins in colorectal cancer play a crucial role in organ tropism. Not only can these molecules mediate highly specific tumour cell adhesion and arrest within the hepatic microcirculation but they can influence and respond to the hepatic environment to stimulate proliferation and metastasis development. Integrin-vascular endothelial growth factor VEGF interactions has been shown to control tumour growth and vascularisation (De *et al.*, 2005).

3.3.2 Tumour Cell Arrest

Tumour cell arrest in the microcirculation of the target host organ is the initial step in metastasis development. Mechanical entrapment (Weiss, 1992;Koop *et al.*, 1995;Naumov *et al.*, 1999;Ding *et al.*, 2001) and cell specific adhesion (Ding *et al.*, 2001;Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) are proposed to explain this. Table 3.2. highlights integrins know to be involved in these early stages of metastasis development.

Mechanical entrapment occurs when the larger tumour cell becomes trapped in the smaller capillaries (Naumov *et al.*, 1999). In haematogenous spread of colorectal cancer, cells would become trapped in the sinusoid vessels with the smallest diameter in the portal circulation. Indeed those in support of mechanical entrapment report finding tumour cells exactly at this location (Naumov *et al.*, 1999;Ding *et al.*, 2001). However

many argue that mechanical entrapment is not a true representation of the metastasis process (Enns *et al.*, 2004;Enns *et al.*, 2005). Mobilisation of the liver during *in vivo* experiments has the ability to disrupt the hepatic architecture, causing vessels within the microcirculation to become compressed. This could be responsible for mechanical entrapment. The type of intravital microscope used for *in vivo* analysis can also cause hepatic distortion. Using the inverted microscope is likely to cause more significant compression of the liver (Enns *et al.*, 2004;Enns *et al.*, 2005).

Table 3.2 The role of integrins in colorectal cancer metastasis development

a. **Metastasis Ability**

Authors	Integrin	Signalling Molecules	Role of integrin and potential therapeutic target
(Bates *et al.*, 2005)	β6	TGF- β	This integrin increases metastatic characteristics and facilitates migration on interstitial fibronectin. This integrin is an important risk factor for early stage disease and novel prognostic disease indicator.

b. . **Colorectal Cancer Cell Invasion/ Motility**

Authors	Integrin	Signalling Molecules	Role of integrin and potential therapeutic target
(Wang *et al.*, 2007)	β3	PI3Kinase/Akt	PRL-3, metastasis associated phosphatase, overexpression is known to increase tumour cell motility and invasiveness. In colorectal cancer, PRL-3 down-regulates β3
(Peng *et al.*, 2006)	α1 β1	Erk1/2	PRL-3 interacts with α1 and decreases the phosphorylation of β1. PRL-3 increased phosphorylation of Erk 1/2.
(Gulubova and Vlaykova, 2006)	α9β1, α5β1		These integrins found to be increased in colorectal cancer cells at invasion front and decreased in primary tumour and liver metatastasis.The expression of these integrins correlated with differential grade of tumour.
(Karadag *et al.*, 2005)	αvβ3, αvβ5		These integrins are found in association with MMP-9. Dentin matrix protein 1 enhances invasion potential of colon cancer cells by facilitating this association.
(Bravou *et al.*, 2006).		Integrin-linked Kinase activates Akt-FKHR pathway.	ILK has been implicated in the development and progression of several human malignancies. In human colorectal cancer, disease progression is associated with activation of this signalling pathway and alterations in β-catenin and E-cadherin
(Mazzocca *et al.*, 2005)	α6β4, α2β1		These integrins are found to associate with ADAM9-Sin colorectal cancer cells. ADAM9-S is able to cleave laminin and promote invasion
(Seales *et al.*, 2005)	β1		Hypersialylation of this integrin in colorectal cancer can increase tumour cell progression. This is achieved by altering cell preference for specific extracellular matrix constituents and stimulating tumour cell migration.
(Deryugina *et al.*, 2004)	αvβ3		αvβ3 integrin is involved in colorectal cancer cell motility *in vitro* and in maliganat invasion and tumour growth *in vivo*. The pro-alphav subunit is converted into the mature alpha v integrin by MT1-MMP.
(Debruyne *et al.*, 2002)		Rac1 and RhoA GTPases	Bile acid was found to stimulate invasion in colorectal cancer through these signalling pathways.
(Jauliac *et al.*, 2002)	α6β4	Nuclear factor of activated T-cells	Promote carcinoma invasion

(Weyant *et al.*, 2000)	Integrins	FAK	NSAIDS and Caffeic acid phenethyl ester, a phenolic antioxidant, reduced colon cancer cell invasion through alter FAK activity. These chemopreventive drugs modulated integrin mediated signalling.
(Daemi *et al.*, 2000)	α6β4		β4 of α6β4 in colon adenocarcinoma may be responsible for signals that stimulate cell motility and tumour invasion. It may also play a role in the expression of MMP-2
(Shaw *et al.*, 1997)	α6β4	PI3 Kinase, Rac	α6β4 increased carcinoma invasion through the PI3 Kinase pathway.
(Chao *et al.*, 1996)	α6β4		This integrin in vitro was found to have ligand independent role in promoting colorectal carcinoma cell invasion.

c. **Colorectal Cancer Cell Target and Arrest**

Authors	Integrin	Signalling Molecules	Role of integrin and potential therapeutic target
(Schluter *et al.*, 2006)			Colon Carcinoma Cells arrest in target organs. The arrest was not due to size restriction. Tumour cell arrest only occurred in metastatic target organs. Migration of colorectal cancer cells corresponded with metastatic potential.
(Enns *et al.*, 2005)	αv spec - αvβ5 integrins		These integrins mediate tumour cell arrest and adhesion, in this *in vivo* model, by the specific interaction between circulating tumour cells and host organ.
(Sato *et al.*, 2003)	αv integrins		These integrins, found to be up-regulated in colorectal cancer lung metastases, may target these cells to lungs.
(Enns *et al.*, 2004)	Integrins		Inhibition of integrins reduced colorectal cancer cell adhesion in the hepatic sinusoids

d. **Colorectal Cancer Cell Adherence**

Authors	Integrin	Signalling Molecules	Role of integrin and potential therapeutic target
(Reyes-Reyes *et al.*, 2006)	α5β1		P-selectin was found to stimulate activation of α5β1 in colorectal cancer cells, through the p38 MAPK and PI3 Kinase pathways, which resulted in creased cell adhesion.
(Enns *et al.*, 2005)	αv spec. αvβ5 integrins		Inhibition of these integrins reduced colorectal cancer cell adhesion. However inhibition of the αv integrins never achieved complete inhibition of tumour cell adherence
(Lohi *et al.*, 2000)	α3β1		This integrin allows for colorectal adenomas and carcinomas to adhere to laminin-5

e. **Colorectal Cancer Cell Migration**

Authors	**Integrin**	**Signalling Molecules**	**Role of integrin and potential therapeutic target**
(Enns *et al.*, 2004)	α2, α6, β1, β4 Integrins		These integrins are involved in colorectal cancer cell migration and extravasation.
(Nejjari *et al.*, 2004)	αv integrins		αv integrins increased colorectal cancer cell migration when inhibition of proprotein convertases occurred.
(Ahmed *et al.*, 2003)	β1	Erk	β1- uPAR complex is responsible for migration/ invasion and plasmin-dependent matrix degradation through MMP-9/MMP-2.
(Sordat *et al.*, 2002)	α3β1	PI3 Kinase pathway	α3β1 integrin expression was twice as high in the low metastatic colorectal cancer cell line. This integrin and its interaction with laminin 5 was found to influence migratory signals and metastatic disease progression.

f. **Colorectal Cancer Cell Microenvironment Interactions**

Authors	Integrin	Signalling Molecules	Role of integrin and potential therapeutic target
(Yazawa *et al.*, 2005)	β1		COX-2 inhibition causes down regulation of β1 integrin. This impairs colorectal cancer cell adherence and migration *in vitro*.
(Brabletz *et al.*, 2004)	β1	Cdx2	Tumour cell environment was shown to influence colorectal cancer cell characteristics. Collagen Type 1, through β1 integrin signalling, causes a down regulation of Cdx2 leading to a loss of differentiation.
(Reinmuth *et al.*, 2003)	αvβ3		S247 αvβ3 antagonist decreases colon cancer metastasis and angiogenesis *in vivo*.
(Roela *et al.*, 2003)	β1		Colorectal cancer cell growth arrest was induced using DMSO. This was found to alter integrin expression and function – affecting tumour cell adhesion and migration. A complex relationship is identified between integrin function and expression and the proliferative state of cells.

Current opinion is in favour of the cell specific adhesion hypothesis (Ding *et al.*, 2001;Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005), which proposes that certain tumour cells have specific cell adhesion molecules that enable them to selectively target an organ. Thus selection of cancer cells occurs as proposed in the second principle of the modified "seed and soil" hypothesis (Fidler, 2003). Organ tropism identified in so many cancers supports this latter theory. Much other evidence supports the cell specific adhesion hypothesis and the role of integrins in tumour cell targeting. Table 3.3 shows the development of *in vivo* models to evaluate early events of colorectal liver metastases. *In vivo* experiments have shown that colorectal cancer cells can be injected by a variety of routes - intra cardiac injection (Enns *et al.*, 2004), intra arterially (Haier *et al.*, 2003;Enns *et al.*, 2005), intra portal (Haier *et al.*, 2003)or intrasplenic (Kemperman *et al.*, 1997) - and still these cells preferentially metastasise to the liver. If the mechanical entrapment theory was correct, these colorectal cancer cells should form metastatic deposits in the capillary beds of the distal systemic circulation post intra arterial injection. Intravenously injected cells would surely have an increased propensity to colonise the lungs not the liver.

This evidence supports the hypothesis that cell surface specific receptors are responsible for organ targeting (Kemperman *et al.*, 1997;Enns *et al.*, 2004;Enns *et al.*, 2005). *In vivo* work, involving the HT-29 human colorectal cancer line, examined which receptors were responsible (Enns *et al.*, 2004;Enns *et al.*, 2005). When specific integrins, especially αv, and the selectin sLe_a were functionally blocked, a significant decrease in the number of colorectal cancer cells targeting and adhering to the liver was observed. Further support for the key role of integrins was obtained from an experiment using green fluorescent protein labelled Chinese hamster ovary cells CHO-K1 (Kikkawa *et al.*, 2002). Wild type GFP-CHO-K1 cells and cells transfected with cDNA

for αvβ3 were analysed. These cells were injected into female Balb/c mice either by the portal vein or the tail vein. Accumulation in the liver or the lungs respectively was detected using intra-vital video microscopy or positron emission tomography. The αvβ3 transfected GFP-CHO-K1 cells showed a significantly higher accumulation in the mouse liver compared to wild type. There was no significant difference in lung accumulation. These results strongly suggest the activated αvβ3 integrin is responsible for the organ specific targeting but would have been strengthened by performing a full proteome analysis, post experiment, of extravasated tumour cells to exclude other variables.

Collectively, this evidence strongly supports the cell specific adhesion hypothesis and in colorectal cancer, shows the integrins are heavily involved in specific organ targeting.

Study	Strain Of Animal	Cancer Cell Line	Molecular Target (Intervention)	Labelling Method	Tumour Inoculation	Outcomes
Steinbauer et al 2003(Steinbauer *et al.*, 2003)	Balb/c mice – SCID and wild type	Murine C26 adeno-carcinoma	none	GFP (Green Fluorescent Protein) and calcein AM	Portal vein	GFP stain in longer experiments can trigger an immune reaction.
Sturm et al 2003(Sturm *et al.*, 2003)	Balb/c mice	Murine C26 adeno-carcinoma	none	GFP	Spleen	Created an animal model for colorectal cancer using murine cancer cells in a murine host.
Haier et al 2003(Haier *et al.*, 2003)	Sprague-Dawley rats	Human HT-29 & rat CC531 colorectal cancer cells	none	Calcein AM	Intra arterially, intra venous and extrahepatic portal vein	Created animal model without GFP label, which could cause immune reaction. Showed despite method of colorectal cancer cell inoculation, cells still metastasised to liver.
Enns et al 2004(Enns *et al.*, 2004)	Sprague-Dawley rats	Human HT-29 cells (HT-29P & HT-29LMM)	Integrins – α1, α3, α5, α6, β4 and α2β1. VCAM-1 and Selectins	Calcein AM	Intra cardiac	Specific integrins play a key role in colorectal cancer cell adhesion to the liver and in tumour migration. ECM of space of Disse is important in metastasis formation.
Enns et al 2005(Enns *et al.*, 2005)	Sprague-Dawley rats	Human HT-29 colorectal cancer cells	Intergins pan αv, αvβ3 and αvβ5	Calcein AM	Intra arterial	αv integrins esp αvβ5 have a key role in colorectal cancer cell adhesion to the liver.

Table 3.3. This table shows the development of *in vivo models* to evaluate the early stages of colorectal liver metastasis. All have used intravital videomicroscopy to analyse metastasis growth and development.

3.3.3 Tumour Cell Adhesion

Recent work (Felding-Habermann *et al.*, 2001;Enns *et al.*, 2004;Enns *et al.*, 2005) shows that tumour cell adhesion within the micro circulation is a key event common to many if not all known human cancer strains. Evidence for this came from *in vivo* real time observation of micrometastasis formation using rat RSC 3 tongue carcinoma cell lines - LM (metastatic) and E2 (non metastatic cells) (Ito *et al.*, 2001). Initial arrest in the sinusoidal vessels was noted for both tumour cell lines. Three days later, the E2 non metastatic cells were completely sheared from the liver sinusoids with no solitary dormant cells observed. A substantial number of the metastatic cells remained. The metastatic cells had demonstrated stable attachment and were subsequently proliferating intravascularly.

Human HT 29 colorectal cancer cells were studied in male Sprague-Dawley rats (Enns *et al.*, 2004;Enns *et al.*, 2005). The αvβ5 integrin, expressed in this cell line, is known to bind avidly to fibronectin. Tumour cell adhesion within the liver was significantly inhibited by Arg-Gly-Asp peptide (RGD) or monoclonal antibodies against αv integrins and especially αvβ5 (Enns *et al.*, 2005). Total inhibition of the adhesion process was never achieved through integrin blocking suggesting other cell adhesion molecules are involved (Enns *et al.*, 2005). Selectins can mediate tumour cell adhesion but in experiments, these bonds appear relatively weak and were readily broken with tumour cells re-entering the circulation (Haier and Nicolson, 2001). Colorectal cancer cell adhesion occurred in patent hepatic sinusoids. Indeed the integrin mediated adhesions were able to withstand haemodynamic pressures (Haier *et al.*, 2003). This phenomenon has also been demonstrated in melanoma and breast cancer. The MDA-MB 435 human breast cancer cells were analysed *in vivo* using severe combined immunodeficient mice (Felding-Habermann *et al.*, 2001). Cells expressing wild type αvβ3 and constitutively

activated mutant αvβ3 were compared. The constitutively activated αvβ3 supported *in vitro* and *in vivo* MDA-MB 435 cell adhesion within the vasculature under haemodynamic flow conditions. An *in vitro* model using melanoma cells demonstrated that activated αvβ3 can mediate cell arrest under haemodynamic flow conditions (Pilch *et al.*, 2002). Integrin activation is vital for adhesion to occur and explains why a poorly metastatic and a highly metastatic strain of HT 29 colorectal cancer, despite having similar integrin expression patterns, exhibit different integrin mediated adhesion patterns (Haier *et al.*, 1999a;Haier *et al.*, 1999b).

The role of integrins in human melanoma cells has been investigated. Two antibodies were raised which were potent inhibitors of melanoma αvβ3 and platelet GPIIb/IIIa integrins (Trikha *et al.*, 2002). These antibodies were shown not to bind to murine integrins. *In vitro*, these antibodies inhibited human melanoma adhesion, migration and invasion – reiterating the crucial role of integrins in adhesion and the other early stages of metastasis development. Interesting results were noted *in vivo*. The antibodies were shown to inhibit melanoma cell lung colonisation in severe combined immunodeficiency mice and inhibit melanoma growth in nude mice (Trikha *et al.*, 2002). In rats, these antibodies react with host endothelial αvβ3 and platelet GPIIb/IIIa as well as tumour integrins. Rat *in vivo* work showed complete inhibition of human melanoma formation and growth. The inhibition of human melanoma growth in the murine host was attributed to functional blocking of tumour αvβ3 integrin. In rats, the complete inhibition was due to the combined blocking of tumour and angiogenic integrins. These results have lead to the development of the CNTO 95 antibody (Trikha *et al.*, 2004), a fully human monoclonal antibody. It inhibits αv integrins and has antitumour and antiangiogenic activity *in vivo*. These results emphasize not only the crucial role integrins play in multi-stages in metastasis development but also their

participation in a diverse range of physiological processes. At the same time, emphasis is placed on the improved efficacy of a multi-targeted approach in the inhibition of metastasis development.

Formation of the preliminary adhesion within the sinusoids is only the beginning of the process and continues until the tumour cell extravasates. Establishment of sustained adhesion is not always successful. If stabilisation can not be achieved, the initial bond can break and the tumour cells re enter the circulation (Haier *et al.*, 2003). Overall however, the targeting and adhesion process is extremely efficient (Chambers *et al.*, 2002). Actin filaments and microtubules within the tumour cells are involved in the stabilisation process – through regulating the flexibility of the cellular cytoskeleton and in stabilisation of the adhesion complex (Korb *et al.*, 2004).

3.3.4 Extravasation

After tumour cell adhesion, extravasation was believed to occur (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). However, this has been questioned. Research by Al-Mehdi in 2000 (Al-Mehdi *et al.*, 2000) has suggested that the metastatic cells can initially proliferate intravascularly (Al-Mehdi *et al.*, 2000;Ito *et al.*, 2001;Sturm *et al.*, 2003). Rat embryo fibroblast cells and a human cell line derived from fibrosaroma were studied *in vivo*. After intravenous injection into immunosuppressed mice, the cells became bound to the pulmonary microvasculature. Subsequently, tumour cells proliferated intravascularly with extravasation occurring very rarely. These findings have since been supported in murine models using the murine C26 colorectal tumour cell line(Sturm *et al.*, 2003) and the RSC 3 rat tongue carcinoma line (Ito *et al.*, 2001). The clinical implications are significant. Intravascularly proliferating cells would be particularly vulnerable and susceptible to chemotherapy. In addition, any therapeutic interventions which interfered with the attachment of these intravascular deposits to the endothelium would prevent establishment of macroscopic organ metastatic colonies. Adversely, such a treatment would produce tumour emboli.

The most recent research involving colorectal cancer suggests that extravasation follows targeting and adhesion (Haier *et al.*, 2003;Enns *et al.*, 2004;Gassmann *et al.*, 2004;Enns *et al.*, 2005). The observed rapid migration into the liver parenchyma (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) protects the cancer cells from haemodynamic stresses. Previous research has demonstrated that fluid shear stress in the physiological range can rapidly kill tumour cells. 70% of B16 murine melanoma cells were killed within one hour (Brooks, 1984). This loss of viability was due to mechanical damage to

the cell membranes at least in part through an oxygen metabolite mediated process (Albertsson *et al.*, 1995).

To extravasate, the tumour cell must possess multiple metastatic phenotypes. The tumour cell must be able to adhere to the target organ but to modify and renew these adhesions in a dynamic fashion. To migrate, the tumour cell may advance through degradation of the local extracellular matrix. The cell must also have the ability to avoid apoptosis and anoikis. Anoikis is the induction of apoptosis due to the lack of cell binding to other cells or to a basement membrane. The process of extravasation is less clearly understood than targeting and adhesion. When colorectal cancer cells were treated with monoclonal antibodies targeting the α6, α2 and β4 integrin subunits, significant inhibition of migration occurred (Enns *et al.*, 2004). The exact mechanism is unknown. The integrins could be involved in colorectal cancer cell extravasation using at least three distinct mechanisms – direct; modification of tumour cell phenotype or enzymatic recruitment and regulation of expression.

A direct mechanism of action has been identified in endothelial cells where the integrin αvβ3 is specifically recruited to the leading edge of lamellipodia during migration (Kiosses *et al.*, 2001). Alternatively, the integrins may induce alteration of cellular phenotypes. In prostate cancer cells (Zheng *et al.*, 2000), the αvβ3 integrin has been shown not only to change affinity for ligands in different functional states but also to activate cell migration by different routes depending on different ligand binding. The αvβ3 integrin was shown to both activate the phosphatidylinositol 3- kinase/ protein kinase B pathway and PI 3-kinase to induce migratory characteristics (Zheng *et al.*, 2000). The αvβ3 integrin again in prostate cancer cells (Manes *et al.*, 2003) has been shown to alter cdc 2 protein and kinase activity levels. Raised cdc 2 levels were found

to increase cell motility - the cdc 2 protein being the downstream effector of the αvβ3 integrin. The αvβ3 integrin transmits signals from the external environment into the cell, modifying the cellular characteristics.

In vitro work in both human dermal microvascular endothelial cells (HMEC) and human breast tumour-derived endothelial cells (B-TEC) further emphasizes the importance of metalloproteinases and integrins. Oxytocin in these cells was found to induce proliferation and migration. A comparison of treated and non-treated cells revealed oxytocin had caused specific matrix metalloproteinases and the integrin β6 gene to be overexpressed (Cassoni *et al.*, 2006). This alteration of migratory characteristics is vital during extravasation. The integrins, while stimulating these changes (Zheng *et al.*, 2000;Manes *et al.*, 2003), participate in proteolytic enzyme recruitment and targeting. The matrix metalloproteinase MMP-2 has been localised to the surface of melanoma cells in association with αvβ3 (Brooks *et al.*, 1996). More recent work in breast cancer (Rolli *et al.*, 2003) has shown a relationship between expression of activated αvβ3 and the production of active MMP-9. In colorectal cancer, the enzyme gelatinase has been identified which may assist with the metastatic colonisation of the liver (Tien *et al.*, 2003). Directing these enzymes to the appropriate cellular location can also be attributed to the integrins. The proteolytically active form of MMP-2 binds directly with αvβ3 in angiogenic blood vessels and melanoma cells *in vivo* (Brooks *et al.*, 1996).

The cellular cytoskeleton plays a key role in regulating cell rigidity and deformability during the early metastatic process (Korb *et al.*, 2004). Integrins can regulate this and may have a key role during extravasation in modifying cell malleability. Constant remodelling of integrin ligand adhesions is necessary. Focal adhesion kinase (FAK)

plays a key role in this dynamic control over the formation, modification and disassembly of adhesive bonds (Von *et al.*, 2005). Focal adhesion kinase has been shown to be directly involved in the early events of tumour cell adhesion under dynamic flow conditions (Von *et al.*, 2005). Experiments have shown that a reduction in FAK expression is associated with decreased cell motility (Hauck *et al.*, 2001). FAK has also been implicated in remodelling of established cell adhesions (Ren *et al.*, 2000) and in adhesion disassembly (Webb *et al.*, 2004).

3.3.5 Apoptosis

Homeostasis is the tight regulation the human body imposes on all physiological processes. The balance between cell proliferation and programmed cell death or apoptosis is no different. Integrins play a key role in this. In endothelial cells, the bonding of αvβ3 with osteopontin causes an increase in intracellular nuclear factor-kappa B (NF-κB) concentration(Scatena *et al.*, 1998). This process mediates endothelial cell survival. Indeed antibodies which disturb this interaction induce cell death. The NF-κB was found to induce osteoprotegerin(Malyankar *et al.*, 2000), a protein that is found to protect osteoblasts and induce apoptosis in osteoclasts(Romas *et al.*, 2002). Endothelial cell survival can also be promoted by α5β1. During angiogenesis *in vivo*, this integrin is found to suppress the activity of protein kinase A. Antagonists of α5β1 activate the protein kinase A enzyme, which leads to activation of caspase-8 and induction of apoptosis (Kim *et al.*, 2002).

The integrins have been shown to activate signalling pathways via the PI-3kinase and MAPK pathways. These pathways can then stimulate growth factor release (Stupack and Cheresh, 2002). However if the integrins are unligated, apoptosis is induced. This

provides a method by which tissue remodelling events can occur determined by the extracellular matrix. The integrins are therefore acting like dependence receptors (Stupack, 2005). This process can even occur in anchored cells if a specific subset of integrins fail to bind their extracellular matrix ligands (Stupack *et al.*, 2001;Kim *et al.*, 2002).

The apoptotic process can be stimulated through a variety of mechanisms. Loss of integrin mediated attachment can trigger apoptosis through activation of caspase-8 (Stupack *et al.*, 2001;Kim *et al.*, 2002), induction of mitochondrial release of Bit 1, a caspase-independent effector of apoptosis(Stupack and Cheresh, 2004;Jan *et al.*, 2004) or through regulation of apoptosis suppressors e.g. Akt/ protein kinase B signalling (Pankov *et al.*, 2003).

So in normal cells, attachment is required for cell survival. This is mediated through integrin pathways. In cells prevented from matrix attachment, a process called anoikis is induced. This is a specific form of apoptosis (Frisch and Screaton, 2001). However in the case of tumour cells, this is lost. These cells are thought to survive in an anchorage independent fashion due to increases in intracellular survival factors e.g. up regulation of Bcl 2 expression and/or loss of p53 activity (Jin and Varner, 2004). Despite this, anoikis has been reintroduced into tumour cells by up regulation of the fibronectin receptor $\alpha 5\beta 1$ by tumour suppressor p16 INK4a (Plath *et al.*, 2000). Cancer cells have a decreased ability to undergo apoptosis. This is especially the case in the early stages of metastasis where over 80% complete the extravasation process (Koop *et al.*, 1995;Luzzi *et al.*, 1998;Chambers *et al.*, 2001). However not all of these extravasated cells progress to form metastasis indeed the metastatic process, post extravasation is highly inefficient.

This is due to dormancy, apoptosis or failure to stimulate angiogenesis (Chambers *et al.*, 2001).

However apoptosis does play a role in the early stages of metastasis especially in therapeutic interventions. Previous work on human melanoma cells has shown a key role for the αvβ3 integrin. Research involving antagonists of αvβ3 blocked melanoma growth by inducing apoptosis (Petitclerc *et al.*, 1999). This research also showed that αvβ3 in M21 melanoma cells, upon binding with denatured collagen, produced a five fold increase in the Bcl 2:bax ratio. The αvβ3 integrin plays a key role in M21 melanoma cell survival.

Further work has looked at how the integrins regulate the apoptotic response to DNA damage through modulation of p53.(Lewis *et al.*, 2002) p53 mediates apoptosis of cells after DNA damage. This includes cancer cells post radiotherapy and chemotherapy. Non adherent cells were found to require cell adhesion to stimulate apoptosis in response to DNA damage. This phenomenon may play a role in therapy resistance. Non adherent cells were found to be killed more effectively by using anti-integrin antibodies or increased p53 or Arf (Lewis *et al.*, 2002).

3.4 Clinical Significance of Integrins For Disease Prognosis

The crucial role of integrins in the early development of colorectal hepatic metastasis has been highlighted and emphasized in this review. The relevance of integrin expression – quality and quantity – in the clinical setting will now be analysed.

The quantity of integrin expression has a key effect on colorectal cancer cell characteristics. In melanoma cells, the metastatic transition of melanocytes to

malignant melanoma has been closely linked to altered αvβ3 expression (Van Belle *et al.*, 1999;Petitclerc *et al.*, 1999;Felding-Habermann *et al.*, 2002). In colorectal cancer, many different integrins have been implicated in the clinical transition and cancer development. Saito et al (Saito N *et al.*, 2007) analysed and compared the expression of various proteins from a primary colorectal cancer cell line and from resultant liver and lung metastases obtained using an *in vivo* model. The ability to metastatise was associated with altered expression in a variety of proteins. Of particular interest was increased expression of β4 integrin. Integrin expression in a different colorectal cancer line was analysed (Sato *et al.*, 2003). Integrin expression in both primary colorectal cancer tumours and the resultant pulmonary metastases were compared. In the pulmonary metastases, a significantly higher integrin αv expression was found. There was no difference in DNA ploidy. Integrin expression influences colorectal metastasis development. In further work, integrin expression was found to vary depending on the tumour environment. In human metastases from both gastric and colorectal cancer, expression of specific integrins α9β1 and α5β1 were found to correlate closely with the histological grade of the tumour (Gulubova and Vlaykova, 2006). Jauliac et al (Jauliac *et al.*, 2002) showed Nuclear Factor of activated T-cells 1 NFAT1 and the recently identified NFAT5 isoform are involved in promoting carcinoma invasion in both human breast and colon carcinoma cell lines. The α6β4 integrin expression was found to closely correlate with NFAT1 and NFAT5 activity. In addition, the clustering of α6β4 integrin was found to induce the transcriptional activity of NFAT5, which increased tumour cells migration.

Comparisons of integrin expression in low and high metastatic cells line have been made. *In vitro*, Haier et al (Haier *et al.*, 1999a) compared low and high metastatic cell lines of the human HT 29 colorectal cancer cell line. The cell lines were found to

express similar integrins. However the different cell lines were found to bind to different extracellular matrix components. Sordat et al (Sordat *et al.*, 2002)analysed 2 cell lines derived from the parent Lovo colorectal cancer cell line – low metastatic E2 and high metastatic C5. Gene expression was analysed. Integrin α3 was up-regulated in low metastatic E2 cells - about 2-fold higher in E2 than in C5 cells. With the use of confocal microscopy, the surface distribution of these intergins was also found to differ between the two cell lines. They concluded that alterations in integrin expression could affect migratory characteristics. The quantity and quality of integrins in colorectal cancer cells can have a significant effect on metastasis clinically.

The role of integrins in angiogenesis and neo-angiogenesis is well established. Integrin αvβ3-VEGF interactions can control tumour angiogenesis and growth (De *et al.*, 2005). The specific role of integrins in colorectal cancer angiogenesis has been examined (Sato *et al.*, 2001;Sato and Miwa, 2002). The integrin expression in 51 patients with colorectal cancer was analysed. 22 patients had lung metastasis, the other 29 had not developed metastases. β3 integrin was found to be expressed in small calibre blood vessels. This integrin was found to be significantly higher in the tumours of patients with lung metastases. β3 expression was lower in the lung metastases than in the primary colorectal cancer tumour. β3 was concluded to be an important vascular endothelial marker for lung metastasis.

Integrin expression has recently been proposed as a prognostic factor in three separate studies. Integrin expression was analysed in 488 colorectal cancer patients. (Bates *et al.*, 2005) β6 was specifically examined. Patients with a high expression of αvβ6 were found to have significant reduction in median survival. This integrin was shown to be a significant risk factor for early stage disease. This integrin was proposed as having not

only a role as a prognostic factor but also as a novel therapeutic target. Hashida et al (Hashida *et al.*, 2002) examined the expression of the integrin α3 in 114 resected colon cancers to establish the clinical significance of this integrin. Patients with low or absent expression of α3 were found to have significantly reduced disease free and overall survival. This integrin was found to be a significant prognostic factor. In an earlier study (Lindmark *et al.*, 1993), α2 and α3 expression in colorectal cancer was studied in 33 consecutive patients. Specifically the expression, distribution and interconnectivity of these integrins with specific ligands were analysed. Loss of expression of these integrins resulted in more advanced disease and reduced survival – suggesting the expression of these integrins could be a useful prognostic indicator.

Most of present knowledge on integrins has been obtained from experiments either *in vitro* or *in vivo*. The above findings however emphasise their clinical significance.

3.5 Discussion and Implications for the Future

This review has focussed on the early stages of colorectal cancer development in the hepatic sinusoids. The integrins are shown to play key roles in each of these stages of tumour cell targeting, adhesion, extravasation and apoptosis not only in colorectal cancer but in other cancers too. This knowledge should enable future therapies to be designed to target integrins as an effective early intervention in the treatment of colorectal liver metastases.

As with any antibody therapy, there are limitations. Exogenously administered antibodies would only have a short therapeutic window before being negated by the immune system. Their therapeutic window would decrease with repeated exposure and

simultaneously the risk of inducing anaphylaxis would increase. Integrin therapies, directed at the early steps of metastasis, are only at an experimental stage. For use in clinical treatment, more research focussed on this area and extensive clinical trials are needed. However this potential treatment would provide significant therapeutic benefit.

To optimise patient outcome, combined therapeutic interventions targeted against metastases are required. Antibodies targeting the early stages of tumour dissemination would be used in combination with present treatment modalities. These antibodies could be designed to specifically target colorectal cancer cells and this specificity would reduce the systemic effects associated with less selective cancer treatment. Antibodies against integrins have the ability to not only impair the early stages of metastasis and significantly reduce colorectal cancer cell adhesion and extravasation, but also simultaneously to render the colorectal cancer cells unable to escape the circulation. The prolonged exposure to haemodynamic shear stresses would cause significant tumour cell mortality. The two most advantageous times to administer this antibody therapy would be at diagnosis or prior to chemotherapy. At diagnosis, antibodies against specific integrins would reduce the risk of metastasis development prior to further management. Prior to chemotherapy, this therapy would optimise and potentiate the oncology regime. Finally, if antibodies targeting specific colorectal cancer integrins were developed, they could be used to deliver selective cytotoxic therapies.

CHAPTER 4

Methods & Materials

4.1. Laboratory Resources

HT29 human colorectal cancer cells and DHD K12 rat colorectal cancer cells were purchased from ECACC (European Collection of Cell Cultures). Media (McCoy's 5A, F10 and Dolbecco's Modified Eagle Media DMEM) and fetal bovine serum (FBS) purchased from GIBCO. Rabbit anti-integrin β4 subunit polyclonal antibody, rabbit anti-human integrin α2 polyclonal antibody and non specific IgG were purchased from Chemicon International. Mouse anti-integrin α6 monoclonal antibody was purchased from Abcam. All Western Blotting equipment was acquired from Invitrogen UK.

4.2.1. Cell Culturing

HT29 cells were cultured in McCoy's 5A media containing 10% FBS and 1% penicillin and streptomycin. DHD K12 cells were cultured in DMEM and F10 1:1 containing 2mM glutamine, 10% FBS and 1% penicillin and streptomycin. Both cell lines were grown and maintained at 37°C in humidified 5% CO_2/ 95% air. The cultured cells were harvested and passaged approximately every 7 days.

When cells reached 70% confluence, the medium was discarded and the cells were washed 3 times with D-PBS. Next, the cells were incubated with 4mls of trypsin at 37°C in humidified 5% CO_2/ 95% air for 5 minutes. When passaging, 10mls of appropriate culture media containing FBS was used. The harvested cell mixture was centrifuged at 300G for 10 minutes. The resultant pellet, after discarding the supernatant, was resuspended in 10 mls of appropriate culture media. Allocated amounts of this cellular

suspension were then added to tissue culture flasks containing 20mls of the appropriate culture media and placed in the incubator at at 37°C in humidified 5% CO_2/ 95% air.

For experiments, the cells were harvested in 10 ml of appropriate media without FBS containing 1% penicillin and streptomycin media. The harvested cell mixture was centrifuged at 300G for 10 minutes. Cells pellets were re-suspended in this media. Cancer cells were labelled with Calcein AM (Invitrogen UK). For experiments involving integrin functional blocking, 1:150 concentration of antibody was added to the cell-calcein AM-suspension one hour prior to animal introduction. Labelled cells were centrifuged at 300G for 10 minutes and the supernatant was discarded. The cell pellet was finally re-suspended in 1.5 mls PBS for animal introduction.

4.2.2. Cell staining

Calcein AM was added to the cell suspension prior to incubation for 2 hours at 37°C. Imaging under the confocal (Fig 4.1) and intravital microscope was performed to determine the concentration for optimal cell staining. At cell concentrations of $1x10^6$ cells per ml, 6μM concentration of calcein AM was found to be optimal.

Fig 4.1.

Fig 4.1.a.

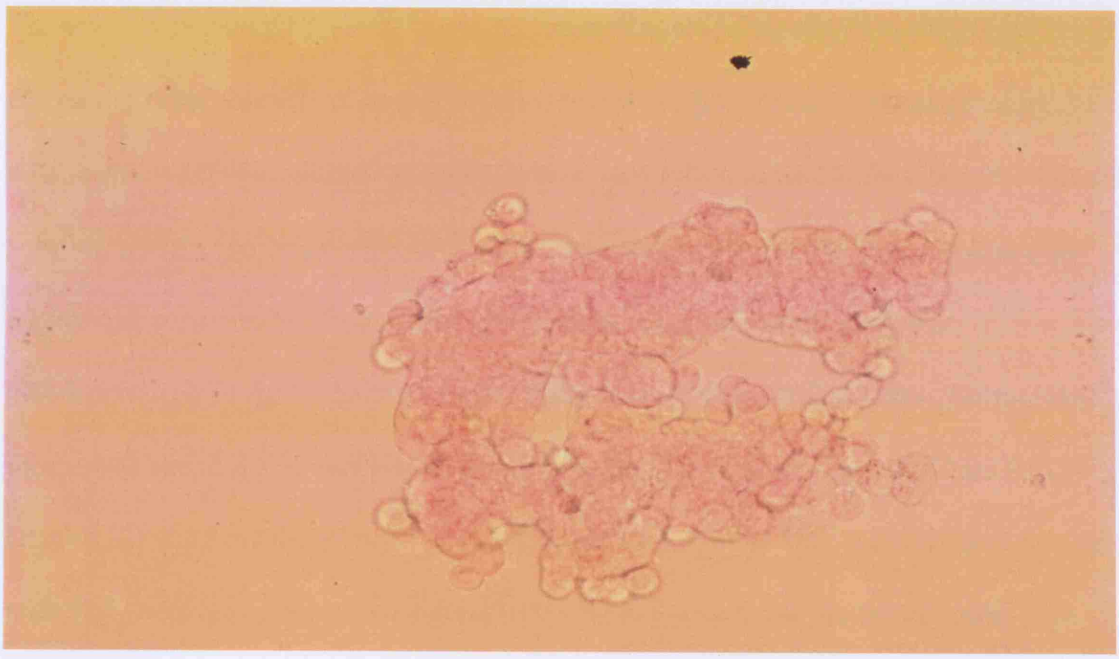


Fig 4.1.b.

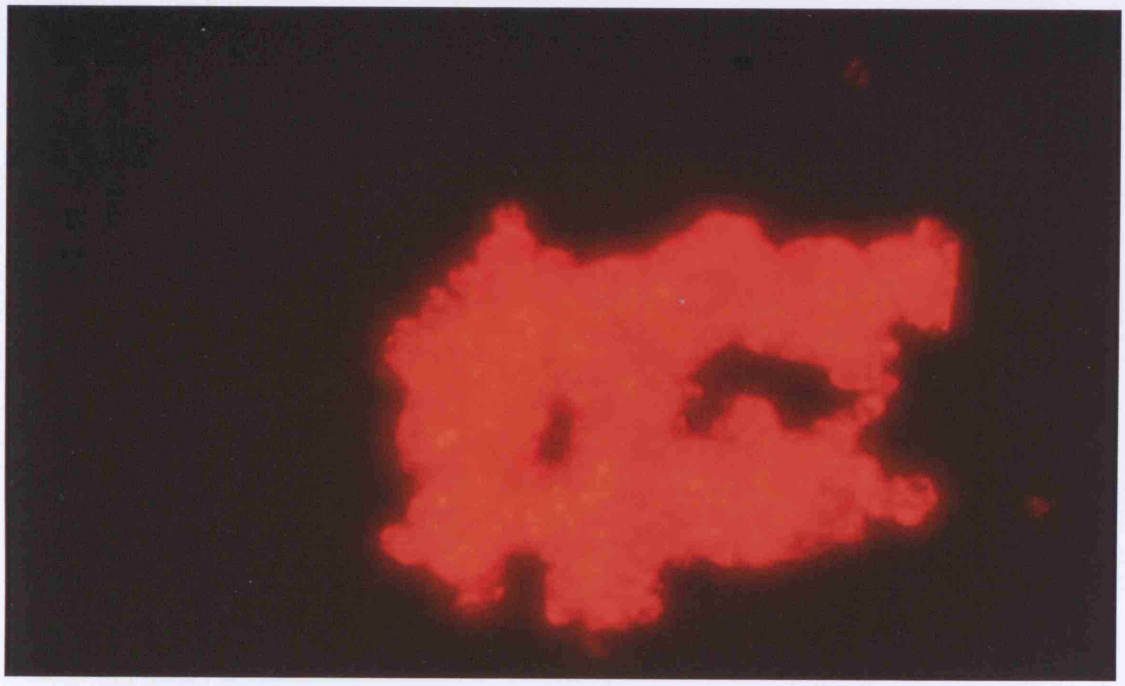


Figure 4.1.a shows DHDK12 cells at a concentration of $1x10^6$ cells /ml on a glass slide. Figure 4.1.b shows the same cell viewed under the confocal microscope. These cells had been labelled with calcein AM at a concentration of 6μM. The cells were left with the calcein AM in an incubator at 37°C for two hours.

4.3. Immunocytochemistry

The initially harvested cells were resuspended in appropriate media containing FBS and antibiotics. The concentration of the cell solution produced was established using the haemocytometer. The optimum concentration was found to be 10^5 cells per ml. The cell solution was placed on an autoclaved slide and incubated at 37°C for three hours to ensure cell adhesion had occurred. Cell fixation was performed using 4% paraformaldehyde for 20 minutes. After three washes with PBS solution, normal donkey serum was added for 30 minutes to block non specific binding. The cell lines (HT29 and DHD K12) were incubated with the antibodies overnight at 4 °C. After several washes with PBS, secondary antibody was added at room temperature for 30 minutes. Two secondary antibodies were used - FITC anti- rabbit and CY3 anti-mouse. Post incubation, the slide was washed in PBS and mounted in Vector shield with Dapi nuclear stain (Fig 4.2).

Fig 4.2

4.2.a

4.2.b

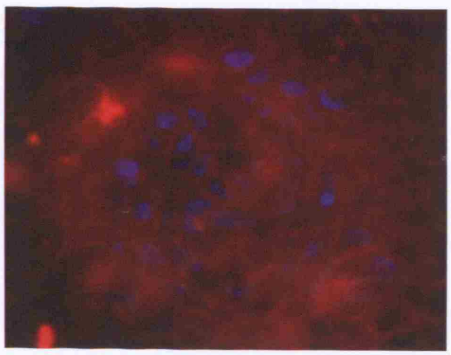


Fig 4.2.a HT 29 β4 integrin stained using FITC anti-rat secondary antibody. Nuclei stained blue with dapi.
Fig 4.2.b HT 29 α6 integrin stained using CY3 anti-mouse secondary antibody. Nuclei stained blue with dapi.

4.4. Western Blotting

Western Blotting Equipment and protocols were obtained from Invitrogen UK. Strict adherence was observed to the manufacturer's protocols.

4.4.1. Preparation – The cells were harvested as described earlier. Post centrifugation the supernatant was discarded. In order to lyse the cells and liberate membrane proteins, HT29 and DHD K12 were resuspended in PBS at a concentration of 2 x 10^6 cells per ml. The samples were stored in -20°C overnight. The following morning, the cells were defrosted and placed in ice. The cell suspension was repeatedly aspirated and expelled through 25 gauge hypodermic needle. 10 µl Triton was added to the solution before further aspiration and expulsion through the hypodermic needle.

4.4.2. Protein Electrophoresis - NuPAGE pre-cast gels were used specifically the 3-8% TA Gel. 10 µl lysed tumour cell samples HT29 and DHD K12 were prepared by adding 2.5 µl NuPAGE LDS Sample Buffer 4x and 7.5 µl deionized water. This solution was heated for 10 minutes at 70 °C. 1x running buffer was prepared by adding 50ml 20xNuPAGE Tris-Acetate SDS Running Buffer to 950ml deionized water. The electrophoresis tank and equipment was assembled according to the manufacturer's instructions. The upper buffer chamber was assembled and inserted into the tank. The upper chamber was filled with 200mls of 1x running buffer. Close observation ensured no leak from this chamber occurred. The lower chamber was filled with 600mls of 1x running buffer. 10 µl of sample was added to each well. The gel was then run at 150V constant for one hour. The current varied throughout the course of the hour – start range 40-55mA; end range 25-40 mA. The effectiveness of protein separation in the first blot was assessed by adding Coomassie Blue, placing the gel on an orbital shaker with ultrapure water and washing repeatedly for a clear background.

4.4.3. Western Transfer - A litre of 1 x NuPAGE transfer buffer was prepared by adding 50ml 20x NuPAGE transfer buffer and 100ml methanol to 850ml deionized water. The blotting pads were soaked in 700ml of 1x NuPAGE Transfer Buffer. The transfer membranes were prepared as recommended by the manufacturer. The filter paper was soaked briefly in 1x NuPAGE Transfer Buffer. The pre-soaked filter paper was placed on top of the gel and air bubbles removed. The plate was turned over in order that the gel and filter paper were facing downwards. Pre-soaked transfer membrane was placed on top of the gel and trapped air bubbles removed. A further piece of pre soaked filter paper was placed on top of the membrane. The two soaked blotting pads were placed into the cathode core of the blot module. The gel/membrane assembly was then placed on the blotting pad in the correct orientation. The gel was closest to the cathode core. The pre-soaked blotting pads were positioned to rise 0.5cm above the rim of the cathode core. The anode core was placed on top of the pads. The blot module was held firmly together and slid into the guide rails on the lower buffer chamber. The Gel Tension Wedge was inserted into the Lower Buffer Chamber and locked into position. The blot module was filled with 1x NuPAGE Transfer Buffer until the gel/membrane assembly was covered. The outer buffer chamber was filled with 650ml deionized water. The lid was placed on the unit and the electrical leads connected to the power supply. Transfer was done using 30V constant for 1 hour. The start current was 220mA and the end current was 180mA.

4.4.4. Chemiluminescent Immunodetection Protocol - The membrane was removed from the gel/membrane assembly. The membrane was then placed in a plastic container with10mls of blocking solution. The container was placed on a rotary shaker set at 1 revolution/sec for 30 minutes. The membrane was then rinsed with 20mls of water for 5 minutes. The water was discarded and the process repeated. The membrane was then incubated with 10mls of 1:1000 primary antibody solution for one hour. The membrane was washed for 5 minutes with 20mls of prepared antibody wash. The antibody was decanted and the gel washed. Washing was repeated three times. The membrane was then incubated with 10mls of secondary antibody solution for thirty minutes. The secondary antibody solution was decanted and the membrane was washed for 5 minutes with 20ml of antibody wash. This was repeated three times. The membrane was washed with 20ml of water for two minutes. After discarding the water, this process was repeated twice. The membrane was then placed on a sheet of transparency plastic but not allowed to dry out. An even application of 2.5ml chemiluminescent substrate was put on the membrane and the reaction allowed to develop for 5 minutes. The excess substrate solution was blotted from the membrane. The membrane was covered with transparency plastic and exposed to an X-ray film for the appropriate time period. The X-ray film was subsequently developed.

4.5. *In vivo* Experiments

4.5.1. Animals - Male Sprague-Dawley and BDIX rats (body weight: 200-250g) were used in the experiment. The study was conducted under the Home Office issued project licence and personal licence in accordance with the UK Government Guidance in the Operation of the Animals (Scientific Procedures) Act 1986. The rats were maintained in a temperature-controlled environment with 12-h light dark cycle and allowed tap water and standard rat chow pellets *ad libitum*.

4.5.2. Anaesthetic - Animals initially were anaesthetized in an anaesthetic chamber with 4L/min of isofluorane (4%) (Baxter, Norfolk, UK). Anaesthetic maintenance was achieved using 1.5-2% isoflurane and 4l per minute of O_2. During anaesthetic, the rats were allowed to breathe spontaneously through a concentric mask connected to an oxygen regulator. Physiological variables (pulse, oxygen saturation, blood pressure and temperature) were continuously monitored during the experiment. The arterial oxygen saturation (SaO_2) and heart rate were monitored with a pulse oxymeter (Ohmeda Biox 3740pulse oximeter, Ohmeda Louisville Co., USA). The animal's body temperature was maintained at 36-37°C using a heating pad (Harvard Apparatus Ltd, Kent UK) and monitored with a rectal temperature probe. Systolic blood pressure was monitored by inserting a polyethylene catheter (0.76mm inner diameter, Portex, Kent, UK) into the right carotid artery. This was connected to a pressure transducer for monitoring of mean arterial blood pressure (MABP). The left jugular vein was cannulated with a smaller polyethylene catheter (0.4 mm inner diameter, Portex, Kent, UK) for the administration of normal saline (1ml/100g body weight/h) to compensate for intra-operative fluid evaporation.

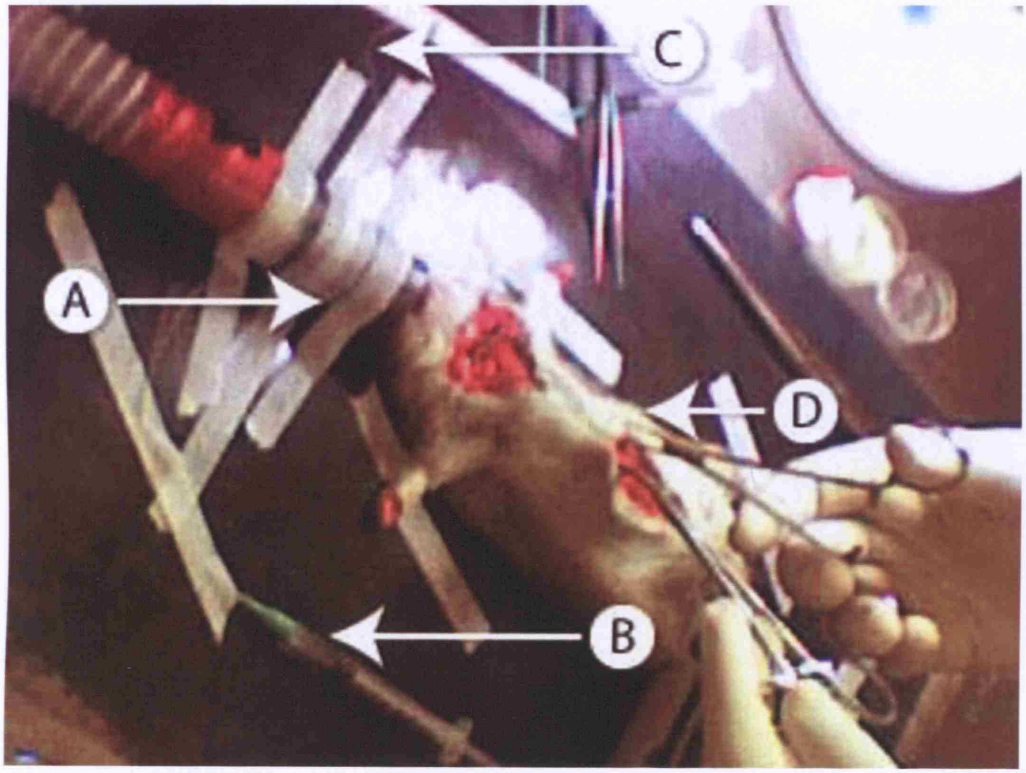
4.5.3. Experiment - A laparotomy was performed through a midline incision. The ligamentous attachments of the liver were cut and the liver was exposed Fig 4.3. The right lobe of the liver was placed on a slide under the intra vital microscope while the rest of the liver was delicately exteriorised to minimise anatomical architectural disturbance (Fig 4.4 a&b). During the experiments, the liver was continuously irrigated with isotonic saline solution and the open abdomen wrapped in cling film to reduce heat loss and fluid evaporation.

For intravital observation of adhesive interactions between circulating tumour cells and the hepatic microcirculation, single cells suspensions ($2x10^6$ cells) were injected intra arterially. The volume was 1.5 ml. Prior to the experiments, sham operations were conducted on three animals. 1.5 ml of PBS solution was not found to cause any significant cardiovascular instability. Haier et al (Haier *et al.*, 2003) have previously shown that intra-arterial injections are associated with fewer systemic complications than intravenous or intraportal administration. The intra-arterial method of introduction was shown to produce the highest number of observed cells within the hepatic microcirculation.

In the experiment, HT 29 cells and DHD K12 cells were used. These two cell lines had been harvested in media without FBS. 2mls of $1x10^6$ cells per ml were incubated at 37^oC in 6μM Calcein AM for two hours. For functional blocking experiments, the cells were in addition incubated with antibody 1:150 concentration for one hour. The cells were then centrifuged at 300G for 10 mins before the supernatant was discarded and the cellular pellet resuspended in 1.5 mls PBS. This cellular suspension was then injected into the carotid cannulae.

Fig 4.3. Experimental Procedure.

The rat is placed in a supine position. Anaesthesia is maintained through an isoflurane/oxygen mixture delivered through a concentric facial mask (A). A midline incision is made in the neck and the right carotid and left jugular are exposed. The carotid artery (B) and jugular vein (C) are cannulated and fluid administered. A laparotomy (D) is performed and the right lobe of the liver mobilised and exteriorised.



4.5.4. In vivo observation of metastatic tumour cell adhesion and extravasation - A 20 microscopic field grid was defined on the exteriorised liver surface using the intra-vital microscope. Filming of the field using the x 40 magnification was performed prior to injection to ensure good views. During filming, images were viewed on a monitor and recorded simultaneously onto DVD (Fig 4.5. a&b). After 1.5 ml of labelled cell solution was injected intra-arterially over 90 seconds, twenty microscopic field grids were filmed at subsequent 15 minute intervals. At each time interval, pulse, blood pressure, temperature and oxygen saturation were recorded. Filming and recording were conducted for a two hour period post injection.

The recordings were analysed post experiment. Total numbers of visualised tumour cell, the number of adhering tumour cells and the number of extravasated tumour cells were counted and recorded. At each time point, the migration rates were calculated with the following formula:

$$\text{Migration rate} = \frac{\text{number of extravasated cells}}{\text{total number of visualised cells}} \times 100\ (\%)$$

4.6. Statistical analysis

Since data were collected serially, analysis of serial measurements was employed. This method is stastistically reliable and arguably more valid for serial measurements (Matthews *et al.*, 1990). One way ANOVA (Graph pad, Prism version 4 2004 edition, USA) with Bonferroni's Multiple Comparison Test was used for statistical analysis and $P < 0.01$ was considered as significant.

Fig 4.4.

Fig 4.4.a. This picture shows the laboratory for *in vivo* work. Visible are the intravital microscope IVVM (A). The video recorder (B) is attached to the top of the IVVM. These are connected to the DVD recorder (C) on the work bench. The images seen under the IVVM are simultaneously displayed on the monitor (D) and recorded onto DVD. Also seen are the computer to analyse pictures (E) and the pulse oximeter (F).

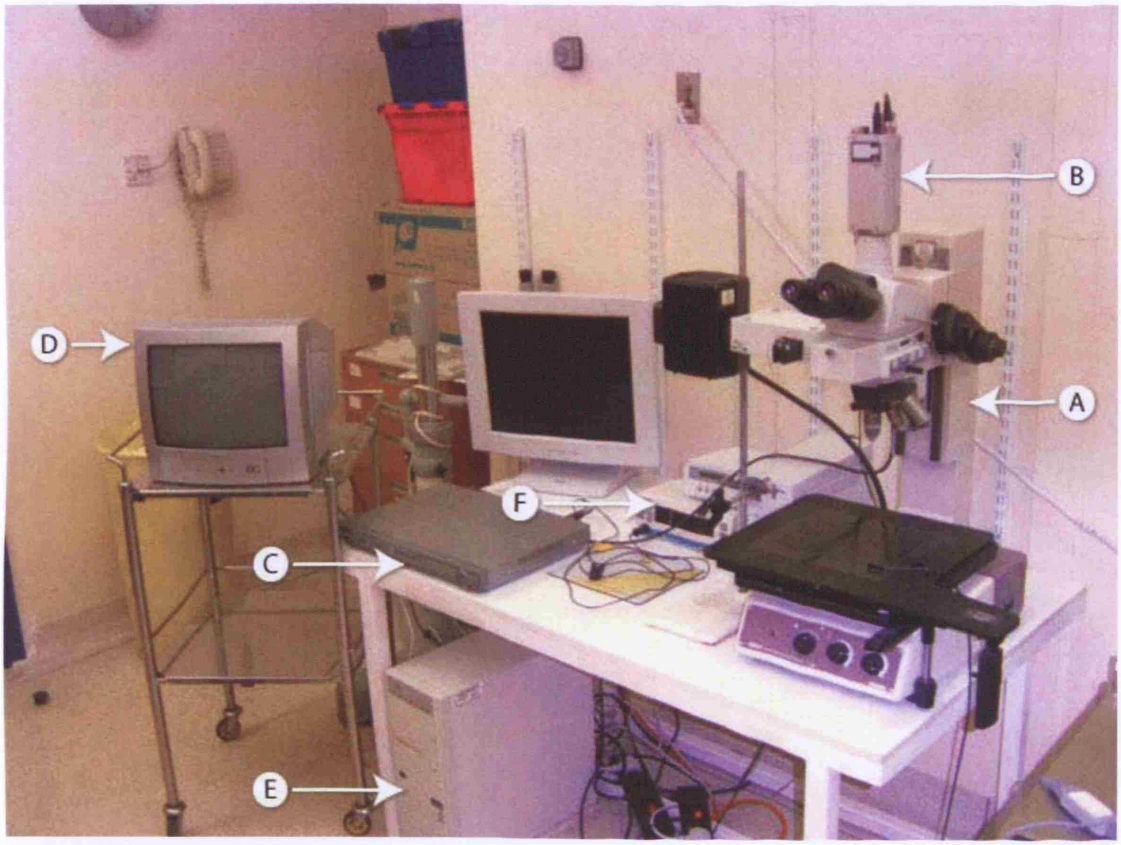
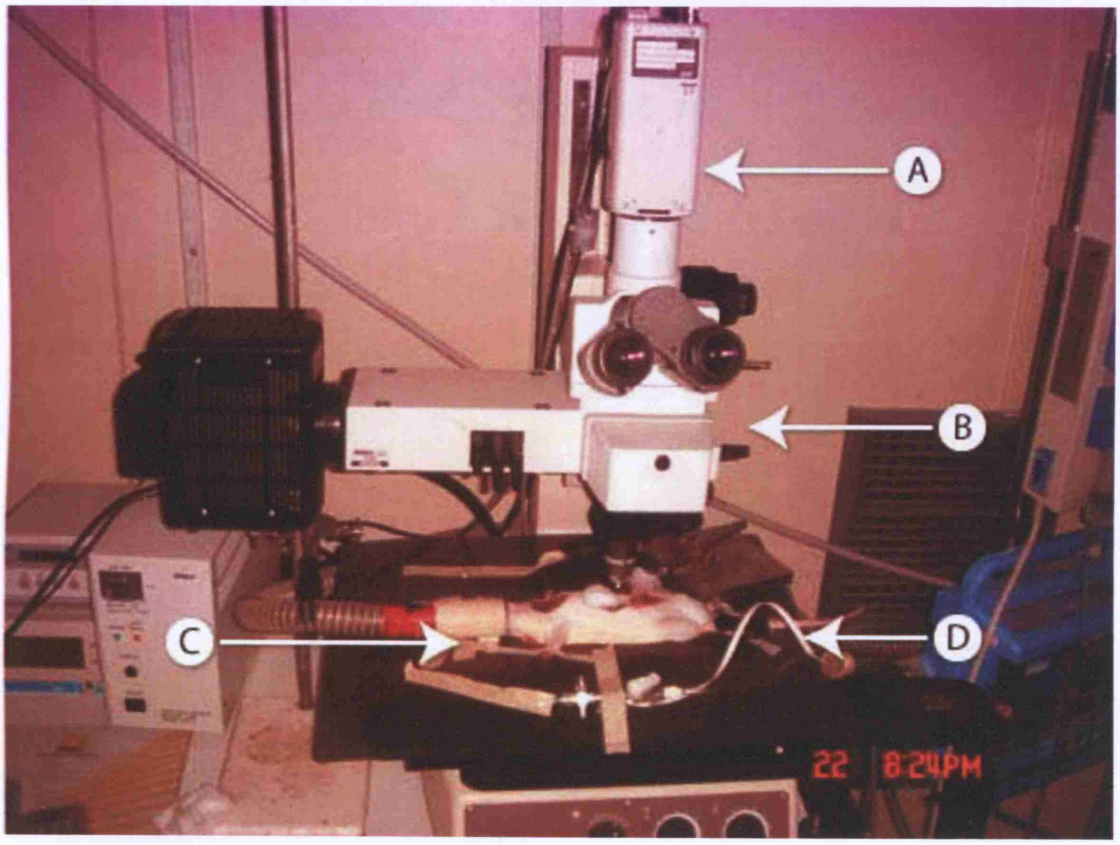


Fig 4.4.b

The rat was placed under the intravital microscope (B) in the supine position. Images were obtained and recorded on the video recorder (A). The right lobe of the liver was exteriorised and placed on the support. During the experiments the rat was anaesthetised. (C) shows the concentric anaesthetic mask used. (D) shows the attachment to the pressure transducer, used to monitor blood pressure throughout the procedure.



CHAPTER 5

A New *In Vivo* Model for the Early Development of Colorectal Liver Metastasis

5.1. Introduction

In order to develop tailored therapeutic interventions, an *in vivo* model for early development of liver metastasis must be established. Such an *in vivo* model should closely mimic the genuine biological cancer metastatic process and allow one to investigate the complex development of metastasis and improve the knowledge of the integral stages involved. This will facilitate the development of novel therapeutic interventions and enable comparison of treated and control groups to analyse the biological effect of the intervention. Numerous models of early colorectal liver metastasis development have been proposed (Table 1.1). Establishment of an *in vivo* model requires tumour cell line and host selection, tumour cell introduction and tumour cell labelling. Each of these processes can influence early colorectal liver metastasis development and affect the biological accuracy of the *in vivo* model.

Arguably the most important decision in establishing a biologically accurate model of colorectal liver metastasis is the compatibility of the colorectal cancer cell line selected and the host. Recent models (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006) have used HT 29 human colorectal cancer cells in a Sprague Dawley rat. The biological accuracy of this model has to be questioned. Despite statistical analysis of human HT29 and rat CC531 colorectal cancer cell lines (Haier *et al.*, 2003) revealing no differences over the thirty minute observation period, the differences between the species in hepatic architecture and homeostatic regulation are likely to distort metastasis development. *In vivo* models using syngeneic cell lines and host are likely to provide a more accurate representation of metastatic development.

A biologically accurate *in vivo* model for colorectal liver metastasis development was established. DHDK12 is a chemically induced rat colon carcinoma cell line induced

from BDIX rats (Martin *et al.*, 1983). Many models studying later stages of metastasis development have used this biologically accurate cancer cell line-host combination (Favoulet *et al.*, 2004;Kobaek-Larsen *et al.*, 2004;Sinibaldi *et al.*, 2004;Benoit *et al.*, 2006;Mezhir *et al.*, 2006). Using Calcein AM as an intracellular label and an intra-arterial method of tumour cell introduction, we compared early liver metastasis development in the four experimental groups - DHDK12 in BDIX, DHDK12 in Sprague Dawley, HT29 in Sprague Dawley and HT29 in BDIX. We established a novel biologically accurate model for the study of early colonic cancer metastasis liver development.

5.2. Methods and Materials

5.2.1 Cell Culturing and Labelling

As described in Chapter 4.2.1 and 4.2.2

5.2.2. Animals

As described in 4.5.1, 4.5.2, 4.5.3 and 4.5.4

5.2.3. Statistical analysis

Since data were collected serially, analysis of serial measurements was employed. This method is statistically reliable and arguably more valid for serial measurements (Matthews *et al.*, 1990). One way ANOVA (Graph pad, Prism version 4 2004 edition, USA) with Bonferrroni's Multiple Comparison Test was used for statistical analysis and $P < 0.01$ was considered as significant.

5.3. Results

5.3.1. The Early Events of Metastasis Development – Experimental Findings

Images from intravital videomicroscopy are shown in Fig 5.1.

Fig 5.1 Intravital Microscope Images

The liver parenchyma has a green autofluorescence labelled (D) in Fig B. The sinusoid vessels are black (E). Three labelled (red) colorectal cancer cells are seen – (A, B & C). (A) is adherent to the sinusoidal walls. The other two (B & C) have migrated out of the sinusoid vessel and into the liver parenchyma

Fig 5.1.a Original Picture from IVVM of rodent hepatic microcirculation.

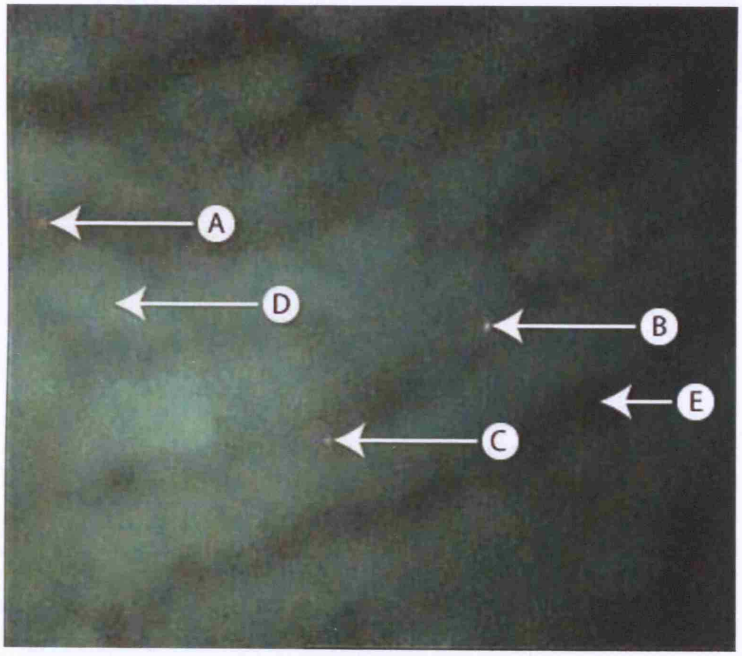


Fig 5.1.b Original picture processed and annotated.

5.3.2 Total number of visualised cancer cells - The total number of visualised tumour cells at each time point is shown in Fig 5.2. The area under the graph (Fig 5.3.) for each group was analysed using the previous reported methods (Matthews *et al.*, 1990). Total number of visualised cancer cells in the DHDK12 in BDIX group was significant higher ($p<0.001$) compared to other three groups. There was no significant difference between the other three groups. Analysis of the maximal cells seen during the experiment was also compared between the four groups (Fig 5.4.). For DHDK12 in BDIX group, the number of maximal visualised cells was significantly higher than in the other groups ($p<0.01$ to $p<0.001$). There was no significant difference in either total visualised cancer cells or maximum visualised cells between the other 3 groups.

5.3.3 Cells adherent to liver sinusoids– The numbers of visualised adherent cells are graphically depicted (Fig 5.5.). Analysis of the area under the graph showed that there were significantly higher numbers of visualised adherent cells in DHDK12-BDIX compared with the other three groups ($p<0.001$). There was no significant difference between the other 3 groups (Fig 5.6.). During the experiment, the number of adherent cells in the DHDK12-BDIX group diminishes markedly in comparison to the other groups. This decrease correlates with the colonic cancer cells moving into the liver parenchyma.

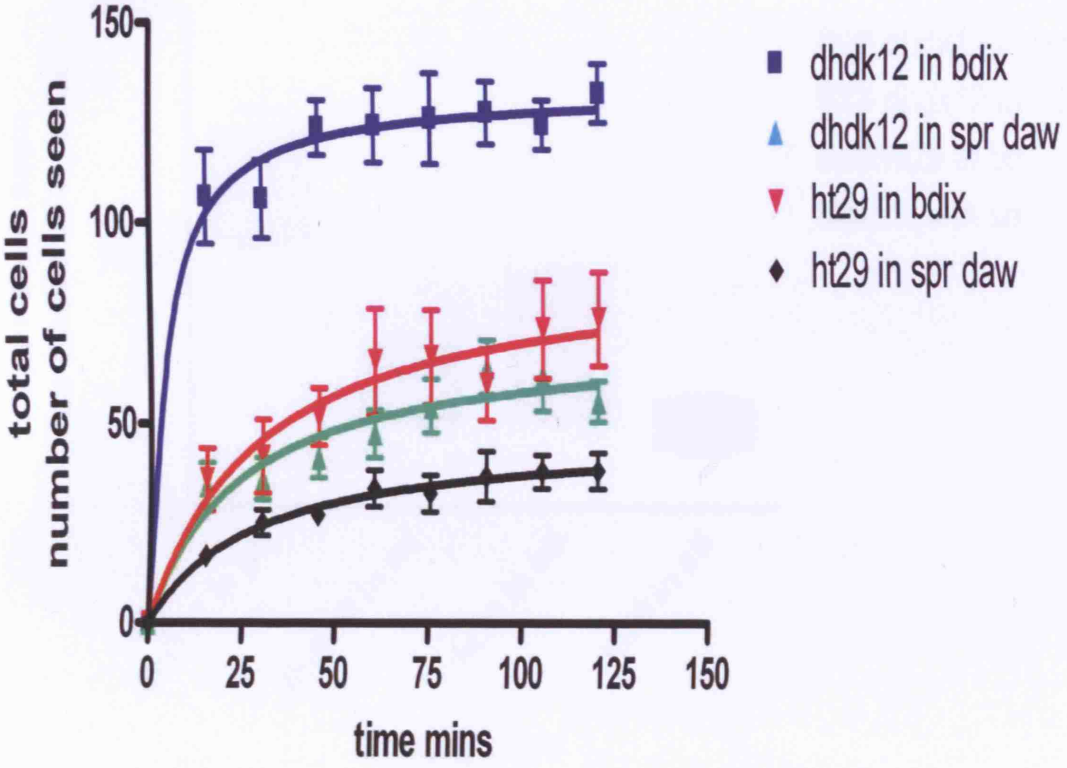


Fig 5.2.
This graph shows the total number of tumour cells visualised in each of the four experimental groups throughout the experiment.

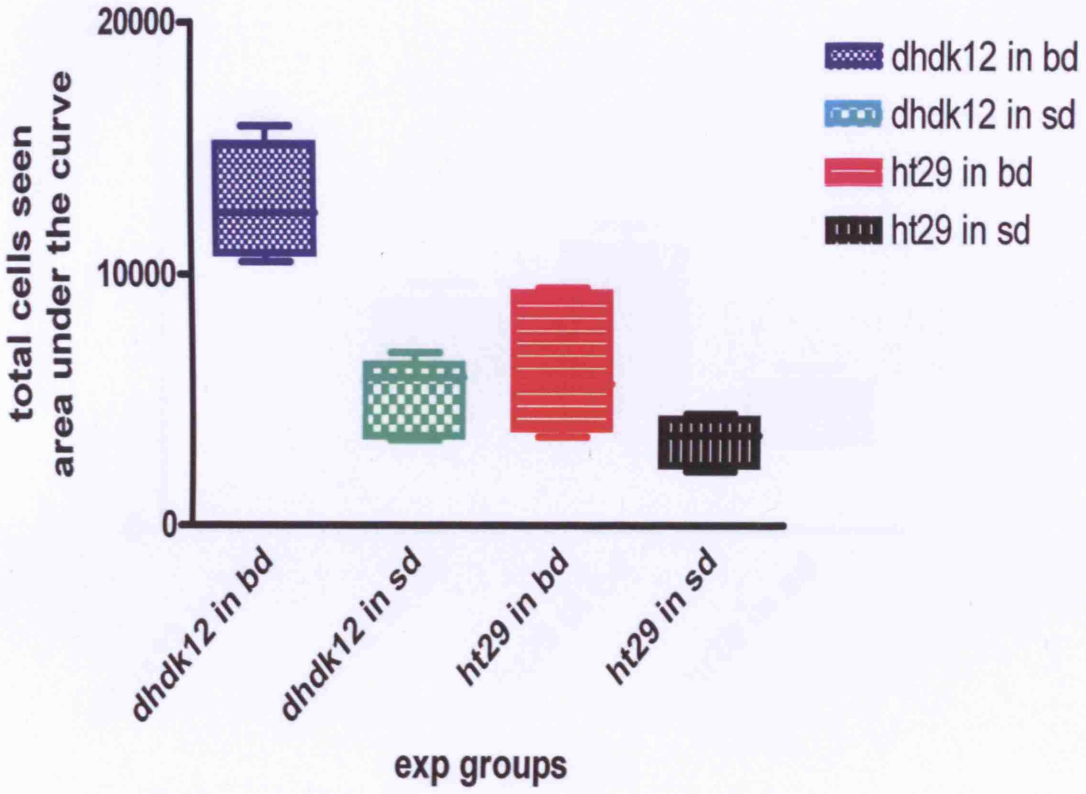


Fig 5.3.
This graphs shows the analysis of the area under the graph for each of the experimental groups. The total number of tumour cells visualised is compared. The number in the DHDK12 in BDIX group is significantly higher ($P<0.001$).

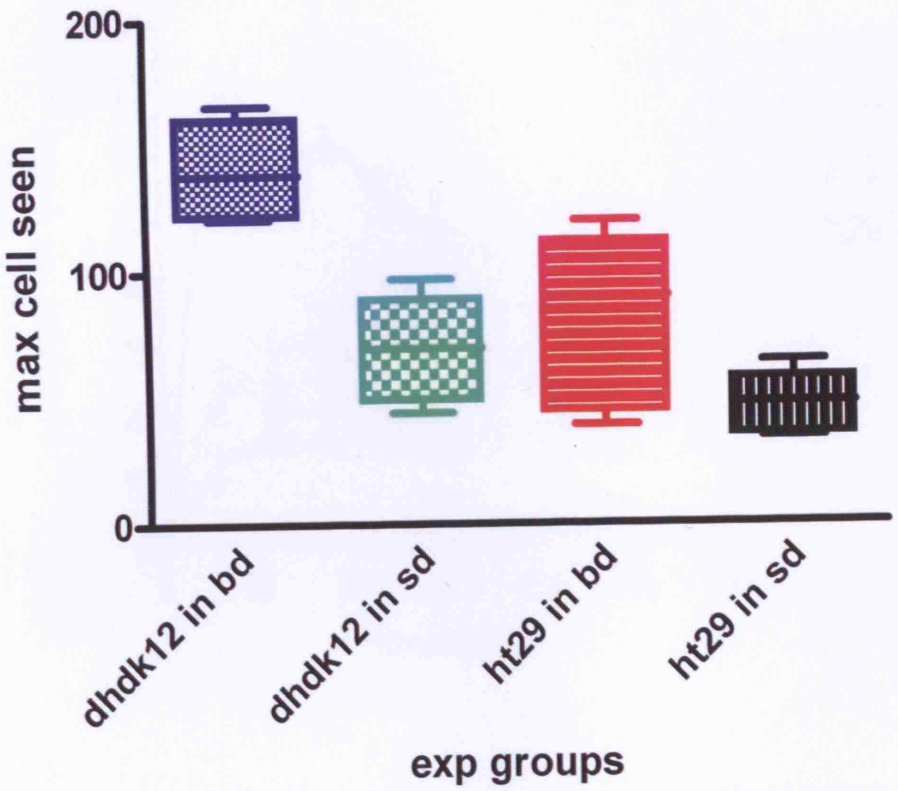


Fig 5.4.
This graph shows the analysis of the maximum tumour cells visualised between the four experimental groups. The number visualised in the DHDK12 in BDIX group is significantly higher (HT29 in BDIX $P<0.01$, DHDK12 in Spr Daw $P<0.001$ and HT29 in Spr Daw $P<0.001$).

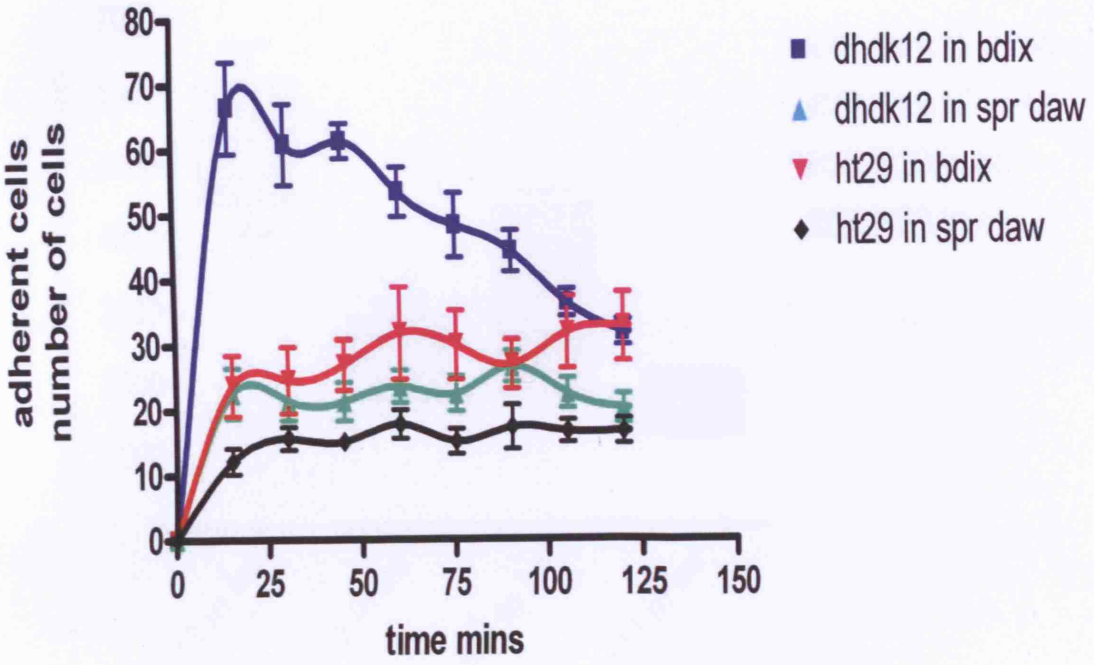


Fig 5.5.
This graph shows the number of adherent tumour cells visualised in each of the experimental groups throughout the two hour experimental period.

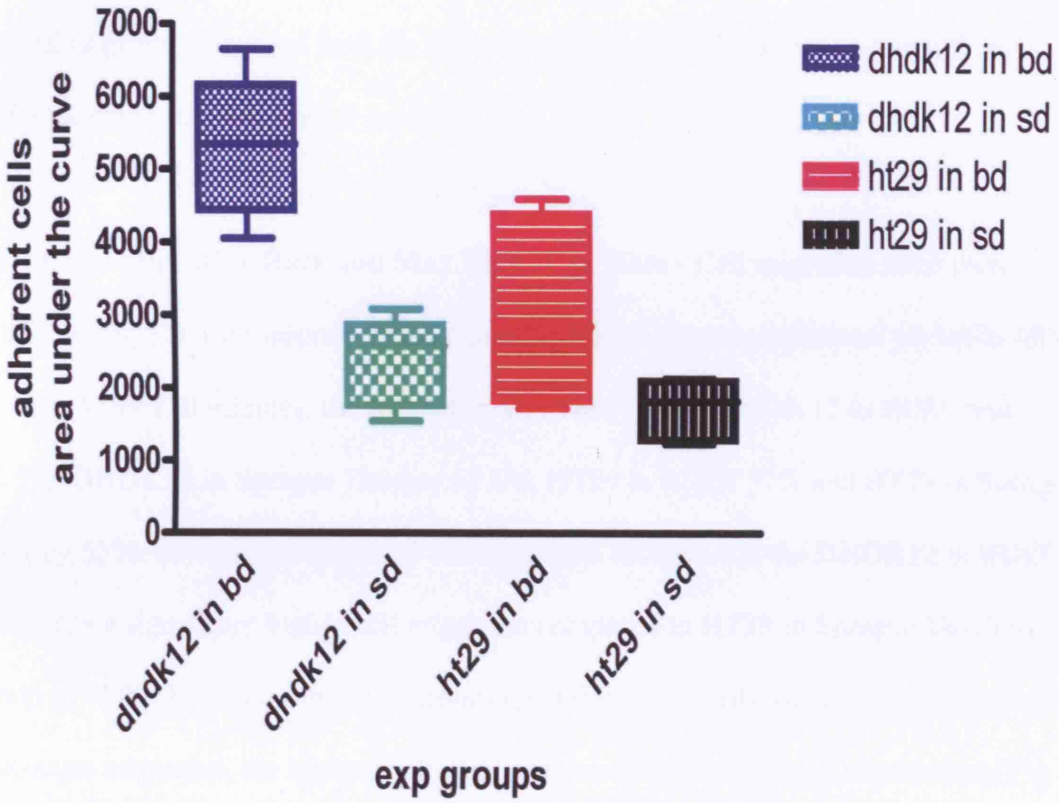


Fig 5.6.
This graph shows an analysis of the number of adherent tumour cells visualised. When statistical comparisons are made between the groups, the DHDK12 in BDIX group is statistically higher (with all groups $P<0.001$).

5.3.4 Cells Extravasated into the liver parenchyma -The extravasation of cancer cells in the four experimental groups was analysed (Fig 5.7). The number of extravasated cells increased in all four groups over the two hour observation period. The increase was more obvious within the DHDK12 cells in BDIX group. Analysis of the area under the graph (Fig 5.8) showed a significantly increased tumour cell migration in the DHDK12 group compared with the other three ($p<0.001$). There was no significant difference between the other 3 groups.

5.3.5 Cells Migration Rate and Max Migration Rate - Cell migration rates were shown in Fig 5.9. Cell migration rates increased during the experimental period in all 4 groups. After 120 minutes, the mean migration rate for the DHDK12 in BDIX was 75.5%, DHDK12 in Sprague Dawley 62.6%, HT29 in BDIX 57% and HT29 in Sprague Dawley 55%. Statistical analysis of cell migration showed that the DHDK12 in BDIX group has a significant higher cell migration compared to HT29 in Sprague Dawley group ($p<0.001$) and the other two groups ($p<0.05$) (Fig. 5.10). In order to compare the maximum migration, the highest migration rate was recorded in each experiment (Fig 5.11). The DHDK12 in BDIX group showed statistically significant increase in maximum migration rate when compared to the other three groups - HT29 in Sprague Dawley and HT29 in BDIX ($p<0.001$) and DHDK12 in Sprague Dawley groups ($p<0.05$) (Table 5.1). For cell migration and the maximum cell migration, there was no significant difference detected between the other 3 groups.

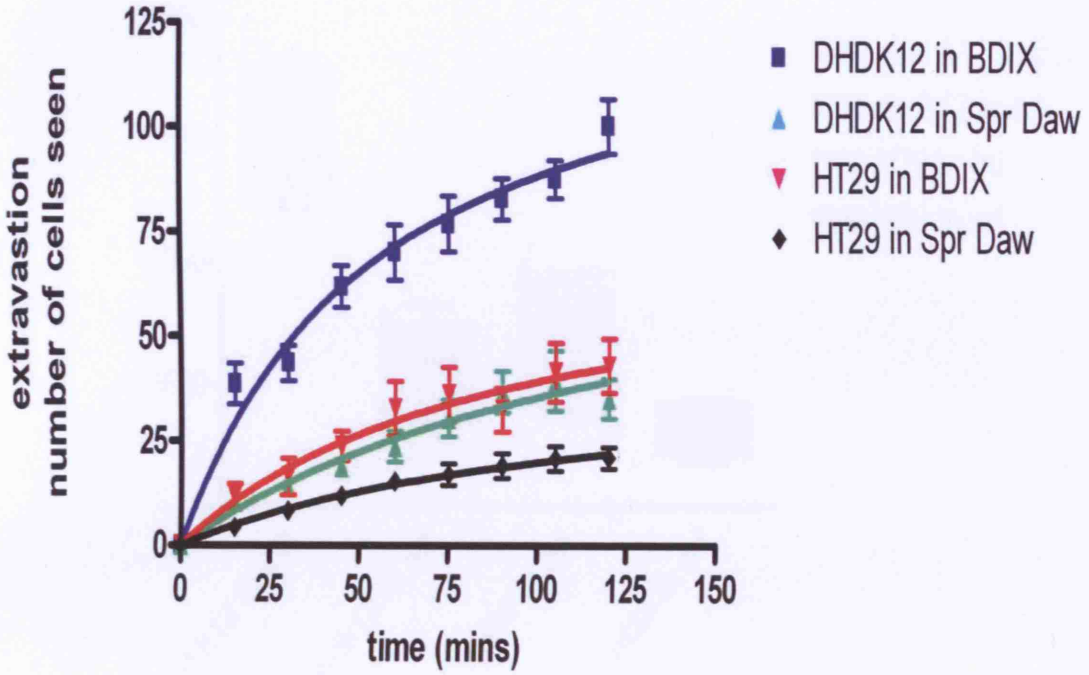


Fig 5.7.
This graph plots the number of extravasated tumour cells detected for each of the four groups during the experiment. The statistical analysis is shown in Fig 5.8.

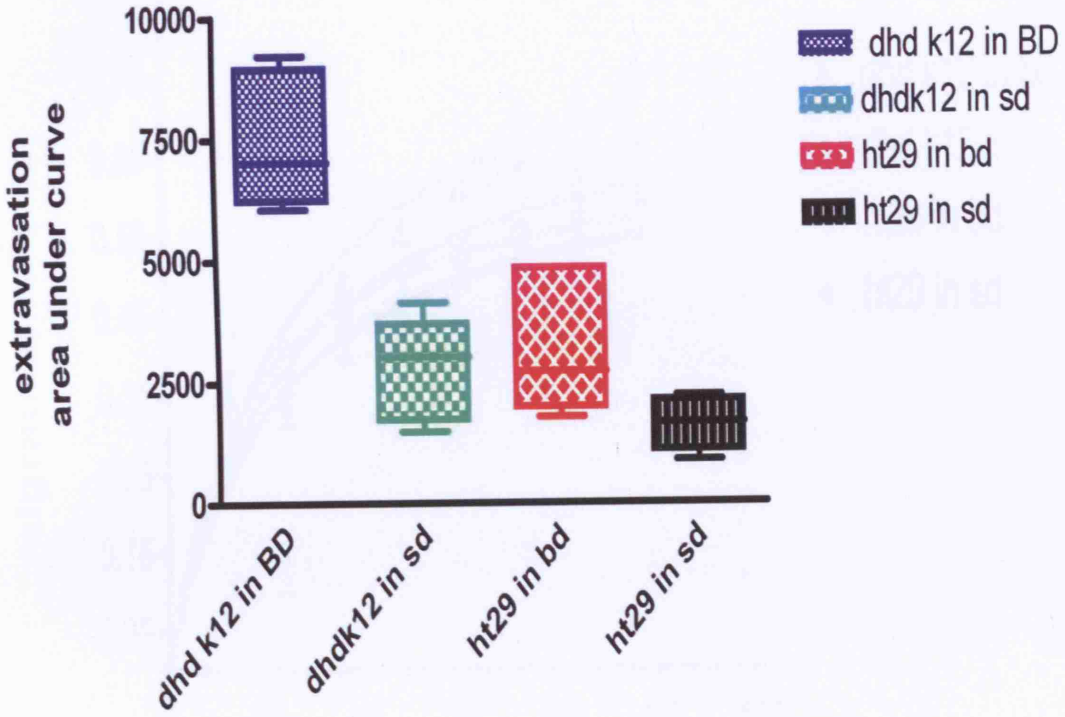


Fig 5.8.
This graph shows an analysis of the number of extravasated tumour cells visualised. When statistical comparisons are made between the groups, the DHDK12 in BDIX group is statistically higher (with all groups $P<0.001$).

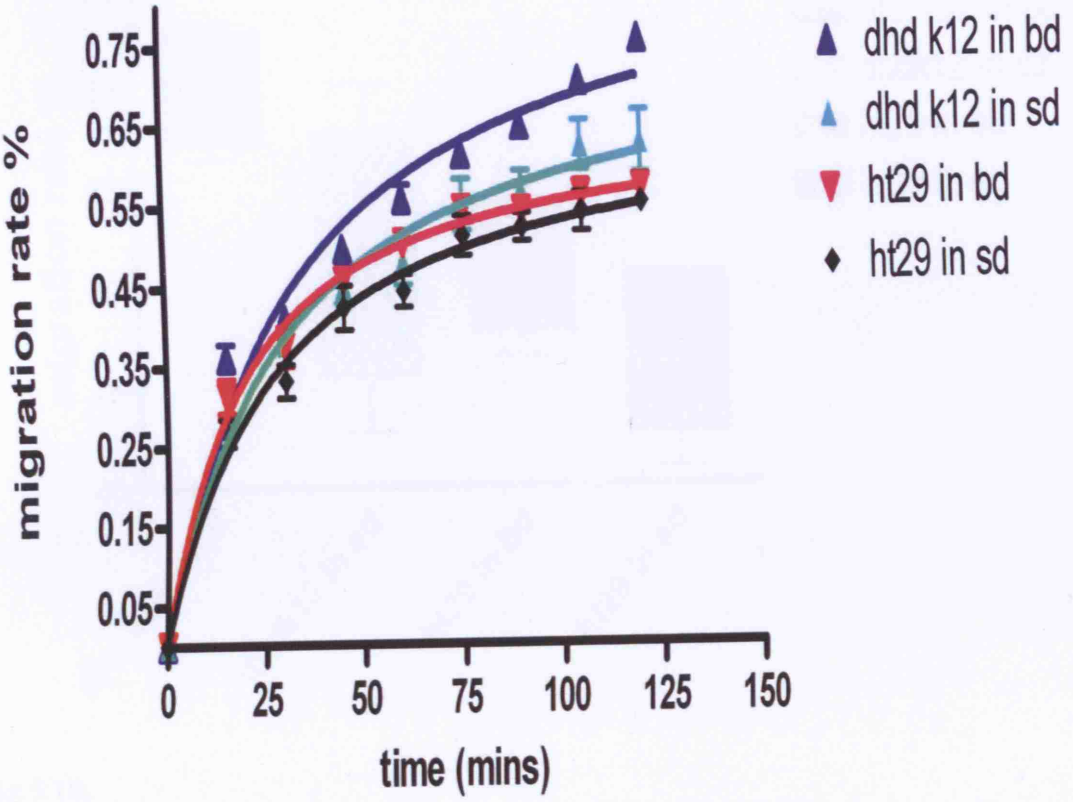


Fig 5.9.
This graph shows the migration rates for each of the four groups during the experiment.

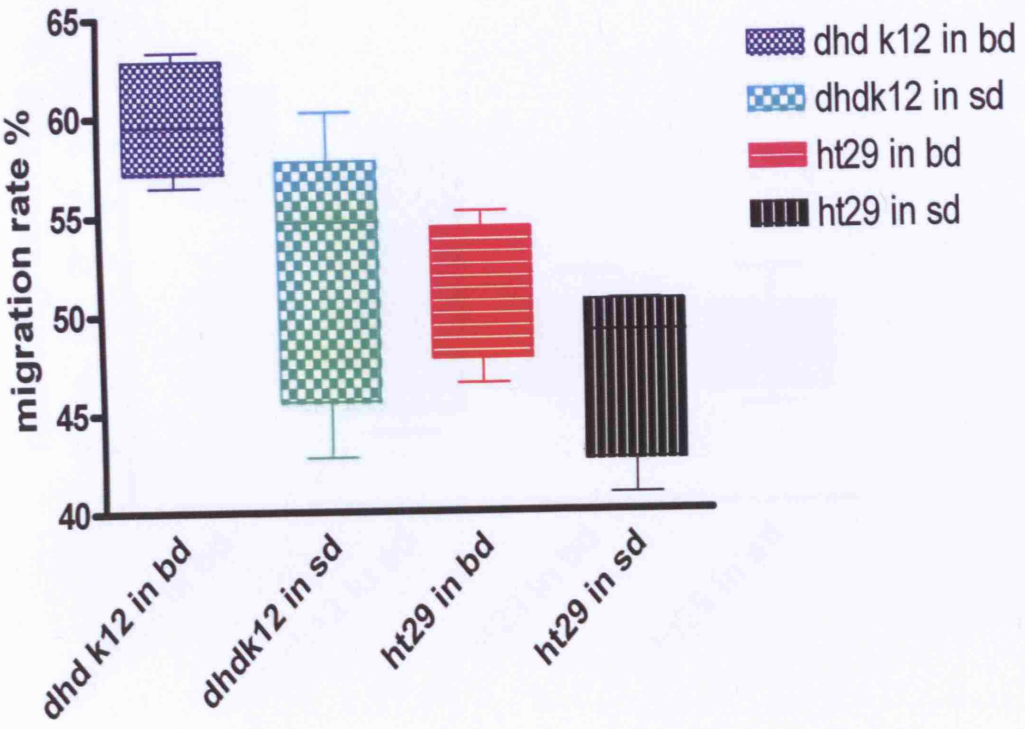


Fig 5.10.
The analysis of migration rates shows that the DHDK12 in BDIX group is again statistically higher (DHDK12 in Spr Daw $P<0.05$, HT29 in BDIX $P<0.05$ and HT29 in Spr Daw $P<0.001$).

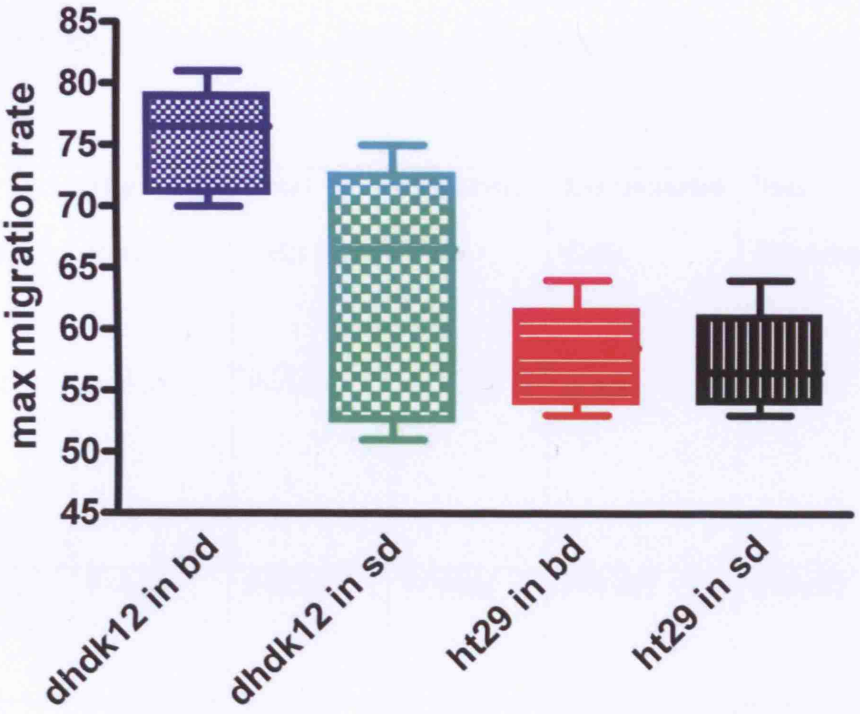


Fig 5.11.
A comparison is made between the maximum migration rate between the groups. The DHDK12 in BDIX group is statistically higher (DHDK12 in Spr Daw $P<0.05$, HT29 in BDIX $P<0.001$ and HT29 in Spr Daw $P<0.001$).

Table 5.1. Statistical analysis of DHDK12 in BDIX vs other experimental groups.

Expt Group	**Migration Rate**	**Total Cells Seen**	**Adherent Cells**	**Extravasated Cells**	**Max Migration Rate**	**Max Cells seen**
DHDK12 in Spr Daw	P<0.05	P<0.001	P<0.001	P<0.001	P<0.05	P<0.001
HT29 in BDIX	P<0.05	P<0.001	P<0.001	P<0.001	P<0.001	P<0.01
HT29 in Spr Daw	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

5.3.6. Systemic Physiological Variables

To study the effect of the experimental procedures, particularly the 1.5 ml intraarterial injection on the systemic variables, two sham groups (SD and BDIX) (n=3) were included (Fig 5.12-5.15). The sham operation was exactly the same as the standard procedure but an intraarterial injection of PBS was introduced instead of the cancer cell suspension. Animal systemic variables such as pulse, temperature, oxygen saturation and blood pressure were measured and recorded. Comparison of the measured variables between the 4 experimental and 2 sham groups revealed no significant differences (Fig 5.16-5.18). The results showed that all animals - 4 experimental and 2 sham groups, 30 rats in total - were physiologically stable throughout the whole experimental period.

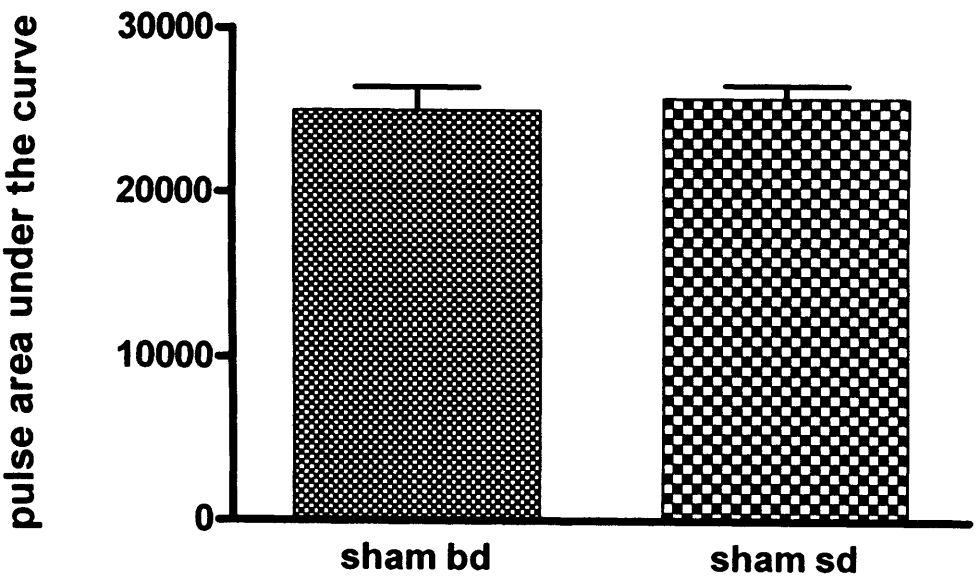


Fig 5.12.
This graph shows an analysis of pulse rates between the BDIX and Sprague Dawley rats. No significant differences were detected.

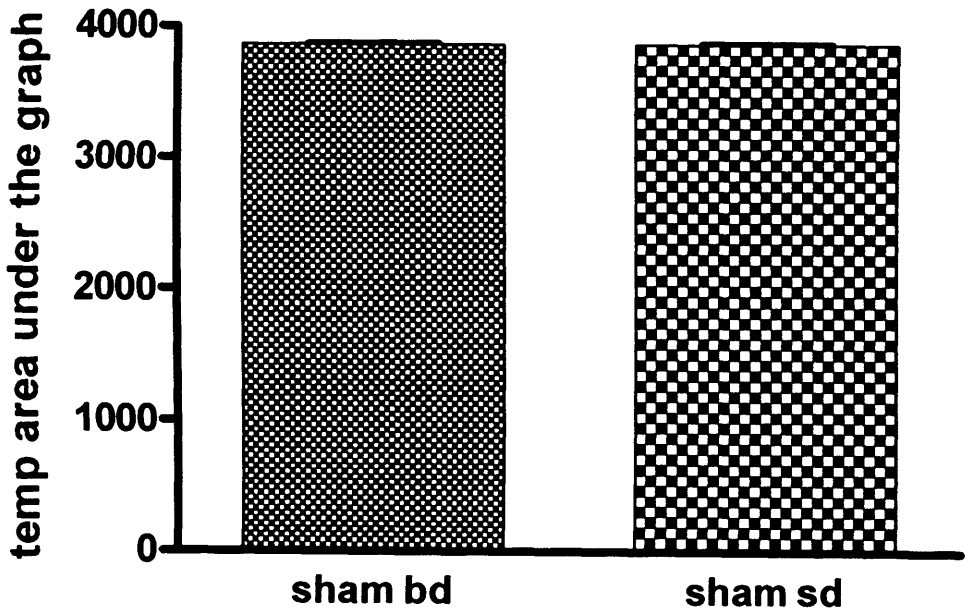


Fig 5.13.
This graph shows an analysis of temperatures measured during experiments between the BDIX and Sprague Dawley rats. No significant differences were detected.

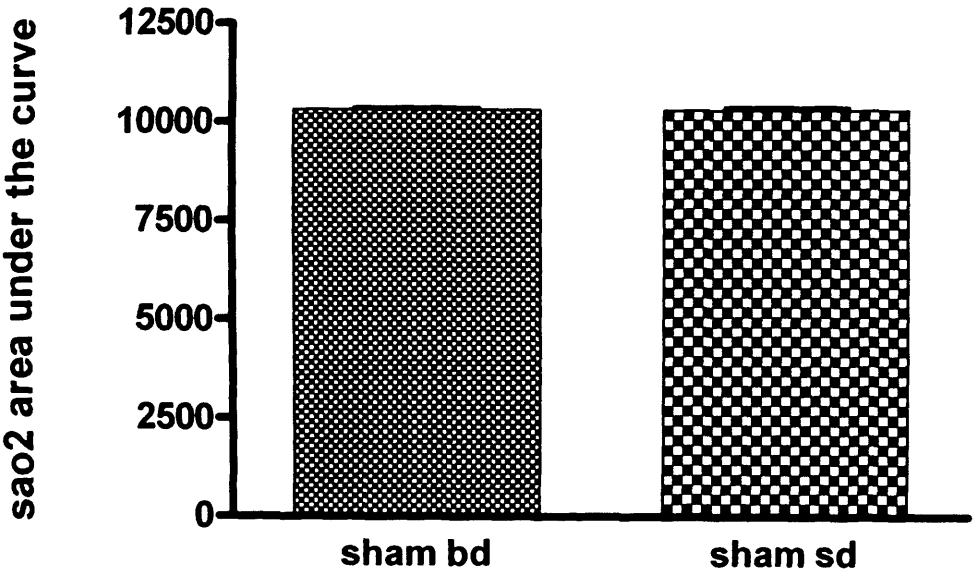


Fig 5.14.
This graph shows an analysis of oxygen saturations measured during experiments between the BDIX and Sprague Dawley rats. No significant differences were detected.

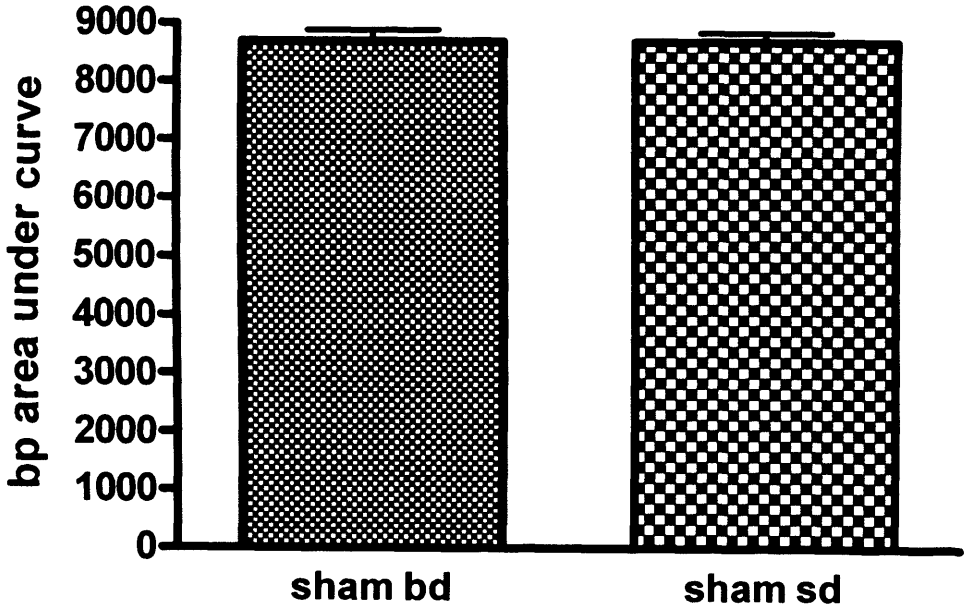


Fig 5.15.
This graph shows an analysis of blood pressure measured during experiments between the BDIX and Sprague Dawley rats. No significant differences were detected.

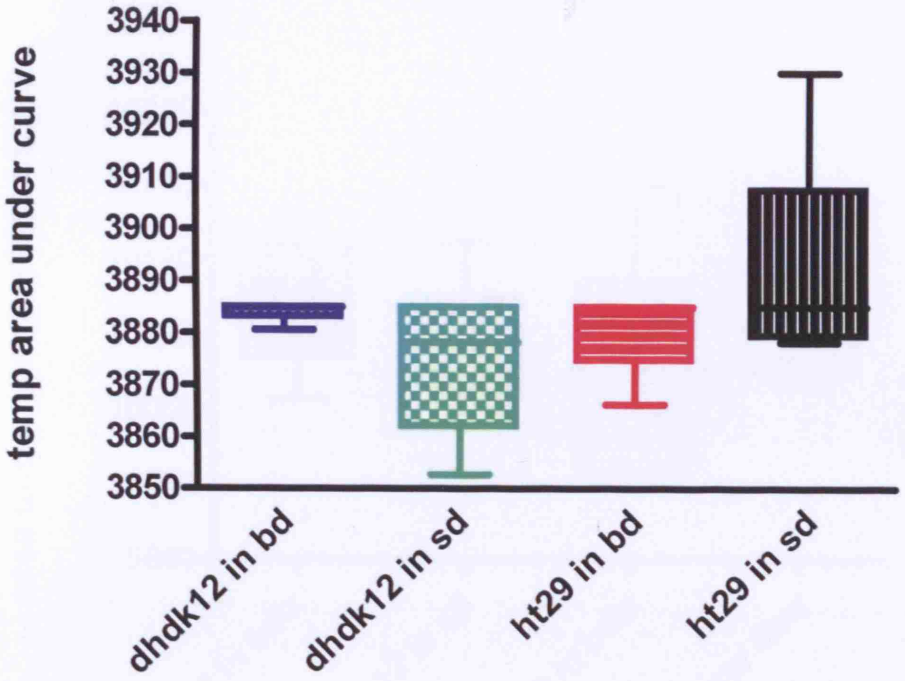


Fig 5.16.
This graph shows an analysis of temperatures measured between the experimental groups. No significant differences were detected.

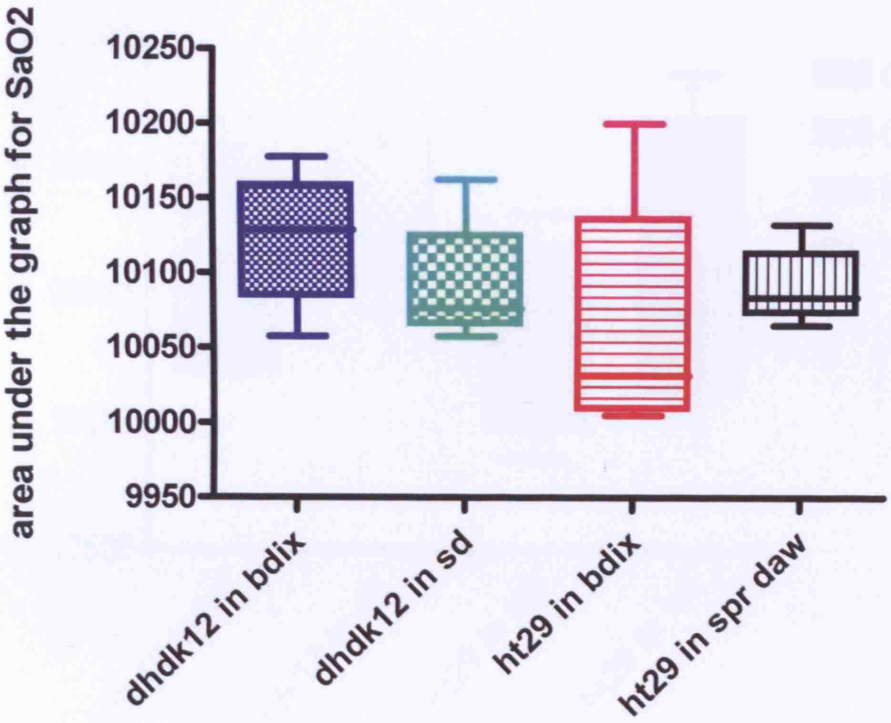


Fig 5.17.
This graph shows an analysis of oxygen saturations measured between the experimental groups. No significant differences were detected.

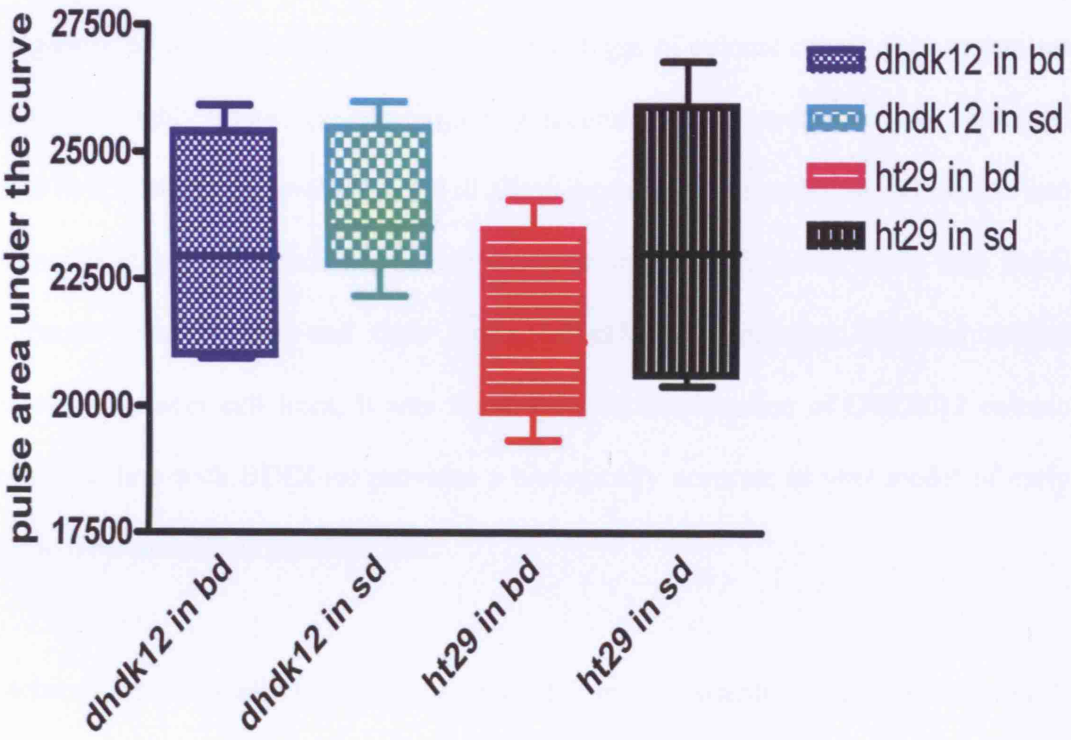


Fig 5.18. This graph shows an analysis of heart rate measured between the experimental groups. No significant differences were detected.

5.4. Discussion

Metastasis development is a highly selective process both locally and systemically. The creation of a biologically accurate model of metastasis is difficult as there are so many introduced variables able to influence metastasis development. Establishment of a biologically accurate model mirroring the early stages of colonic cancer liver metastasis initiation is highly important. A biologically accurate *in vivo* model for early colorectal cancer liver metastasis development will allow increased understanding and insight into the crucial stages in metastasis development. It will also facilitate study into novel therapeutic interventions and their biological effects. Comparing different animal species and cancer cell lines, it was found that the combination of DHDK12 colonic cancer cell line with BDIX rat provides a biologically accurate *in vivo* model of early colonic liver metastasis development.

To achieve a biologically accurate *in vivo* model, many variables should be minimised. The labelling process of colorectal cancer cells can produce high levels of background fluorescence which can limit accurate analysis of metastasis development (Weissleder *et al.*, 1999;Hoffman, 2002). The administration of exogenous reagents (Sweeney *et al.*, 1999); cellular labelling (Steinbauer *et al.*, 2003); surgical techniques, anaesthesia and the intra-vital microscopy procedure can all distort the image of metastasis development (Enns *et al.*, 2004) and affect the biological accuracy of the *in vivo* models. Arguably, the biggest factor influencing the accuracy of metastasis development is cancer cell line-host selection and compatibility. Therefore, the cancer cell-host selection and compatibility was decided to be the focus of the experiment.

The DHDK12 colonic cancer cell line was chemically induced in the BDIX strain of rat (Martin *et al.*, 1983). Therefore, the *in vivo* model of metastasis, DHDK12 cell line in the BDIX rat, should be biologically compatible. Comparison of summary results of this group, with the other three experimental groups, showed a significant increase in every measured experimental parameter of metastasis development - total cancer cells visualised within the hepatic microcirculation, cancer cells adhering to the sinusoids, cancer cells extravasating, migration rate, maximum migration rate and maximum total cancer cells visualised. No significant differences were noted between the three other groups. No haemodynamic variables were noted between the two rat strains in the sham experiments. No significant haemodynamic variables were evident between the four experimental groups. These results demonstrate that cancer cell line-host selection and compatibility has significant effects on early metastasis development. Previous work (Chambers *et al.*, 2001;Chambers *et al.*, 2002;Onn and Fidler, 2002;Fidler, 2002;Fidler, 2003) has highlighted the importance of tumour cell interaction with both the organ of metastasis development and with local host homeostatic mechanisms. These findings suggest not only does non-related cancer cell line-host combination affect metastasis development but that the introduction of colonic cancer, from a different strain within the same species, alters metastasis development. These results question the biological accuracy of current *in vivo* models focussing on the early stages of colorectal liver metastasis development which have predominantly used human HT29 colorectal cancer cells in a rat host (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006).

The influence of cancer cell line-host selection has been studied in other metastasis models. Using orthotopic tumour models, human colorectal cancer liver metastasis development was examined in female BALB/c mice (Flatmark *et al.*, 2004). 12

colorectal cancer cell lines were implanted and showed considerable variation with regard to tumour propagation and dissemination. Only one, of the twelve colorectal cancer cell lines, produced liver metastases. This occurred in only two of ten animals. While the method of colorectal cancer cell introduction was different – orthotopic versus haematogenous – cancer cell line-host selection has a significant effect on tumour development. In most hosts, immunosurveillance and clearance is occurring. A further study looking at HT29 human colorectal cancer cells produced similar results in immunocompetent mice. However in the immunocompromised SCID mouse, significantly higher rates of lymph-node metastases and liver and lung metastases occurred (Guilbaud *et al.*, 2001).

In this *in vivo* model, the principle stages of metastasis development were similar to those described by Haier et al (4). Tumour cell arrest and adhesion in the hepatic microcirculation was evident in patent hepatic vessels. This further supports the tumour cell specific adhesion theory (Ding *et al.*, 2001;Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). This theory proposes that certain tumour cells have specific cell adhesion molecules that enable them to selectively target an organ.

Establishment of sustained adhesion was not always successful. Occasionally, tumour cell stabilisation was not achieved and the initial bond broke with the tumour cells re entering the circulation. Formation of successful adhesion was swiftly followed by extravasation. Previous work by Al-Mehdi et al (Al-Mehdi *et al.*, 2000) had suggested that metastatic cells initially proliferated intravascularly (Al-Mehdi *et al.*, 2000;Ito *et al.*, 2001;Sturm *et al.*, 2003). In this model, cancer cells were found to rapidly migrate into the liver parenchyma. By the end of the two hour observation period, over 55% of cancer cells in all four groups had extravasated.

In conclusion, DHDK12 colonic cancer cell line haematogenously introduced into a BDIX rat provides a biological accurate *in vivo* model of early colonic liver metastasis development.

CHAPTER 6

Detection of Key Integrins In Different Species of Colorectal Cancer

6.1 Introduction

Many studies (Sturm *et al.*, 2003;Haier *et al.*, 2003;Steinbauer *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006) have investigated the early stages of colorectal cancer metastasis to the liver and the key molecules involved. A recent series of studies (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005) have focussed specifically on colorectal cancer cell arrest and adhesion in the liver and subsequent extravasation. Haier et al (Haier *et al.*, 2003) used human HT29 colorectal cancer cells labelled with Calcein AM and introduced these tumour cells into the circulation – mimicking haematogenous dissemination. Integrin expression, in the human HT29 colorectal cancer cell line, has previously been characterised (Haier *et al.*, 1999a). Despite various methods of tumour cell introduction - intra-cardiac (Enns *et al.*, 2004), intra-arterial(Enns *et al.*, 2005), intra-portal and intra-venous(Haier *et al.*, 2003) - the colorectal cancer cells targeted and adhered to the liver. No tumour cell rolling was observed and tumour cells were found to adhere to the microcirculation vessel walls despite the vessels remaining patent. The integrins were identified as playing a key role in this process specifically the αv integrins (Enns *et al.*, 2004;Enns *et al.*, 2005). Particular focus has been placed on the role of the integrins in cancer development and dissemination (Eble and Haier, 2006). Alteration of integrin expression has been shown to be associated with cancer cell development in the transition of melanocytes into malignant melanoma (Van Belle *et al.*, 1999). Activated αvβ3 has been shown to play a critical role in conveying the metastatic phenotype in breast cancer cells (Felding-Habermann *et al.*, 2001;Felding-Habermann *et al.*, 2002).

In the experiments by Enns et al, functional blocking of specific integrin subunits α2, α6 and β4 was found to reduce tumour cell migration into the hepatic parenchyma (Enns *et al.*, 2004). In this preliminary study, the expression of these three integrin subunits α2, α6 and β4, in human HT29 and rat DHD K12 colorectal cancer cells, was examined. The techniques of Western Blotting and Immunocytochemistry were used.

6.2 Methods

6.2.1 Materials and Cells

As described in 4.2.1.

6.2.2 Immunocytochemistry

As described in 4.3.

6.2.3 Western Blotting

As described in 4.4.

6.3 Results

6.3.1 Immunocytochemistry

The results of the immunocytochemistry (Fig 6.1.) show the three integrins are expressed in both the rat DHDK12 and human HT29 colorectal cancer cell lines.

Integrin - subunit	HT 29	DHD K12
α2	++	+++
α6	+++	++
β4	+++	++

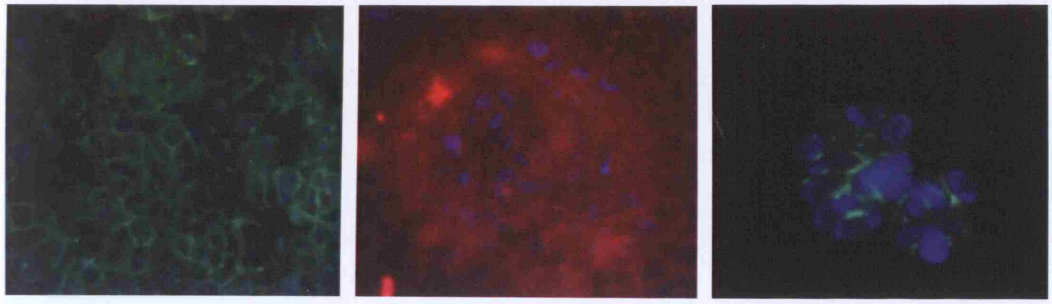
Table 6.1.

This table quantifies the immunocytochemistry observed. For each integrin in each cell line, a score of +, ++ or +++ has been assigned.

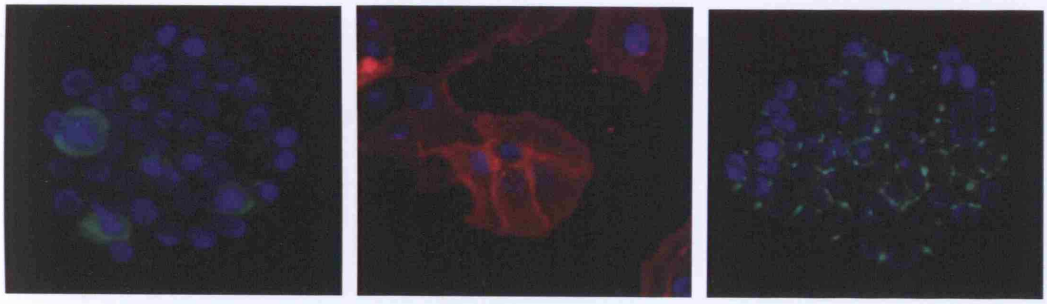
In the microscopic fields:-

- + is defined as 50-70% of cells viewed stained positive for the integrin
- ++ defined as 70-90% of cells viewed stained positive for the integrin
- +++ defined as greater than 90%. of cells viewed stained positive for the integrin

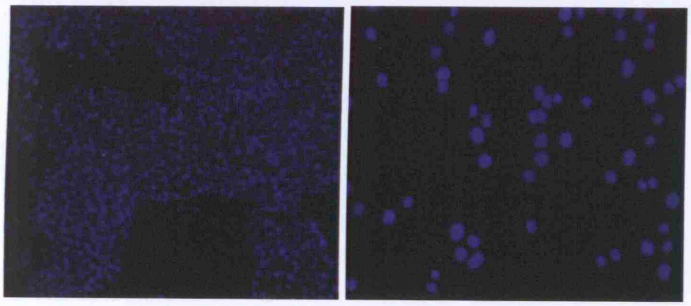
The results obtained for our HT29 cell line (Table 6.1) are similar to the previous phenotypically different HT29 cell line. However, slightly diminished α2 expression was noted (Haier *et al.*, 1999a). Immunocytochemistry analysis supports strong expression with over 70% of cells staining positive for each specific integrin. The DHDK12 cells exhibit strong expression of the α6, α2 and β4 integrins. The α2 integrin seems to be particularly well expressed in this cell line with over 90% of cells viewed staining positive, slightly more than the other two integrins.



A. HT 29 β4 B. HT 29 α6 C. HT 29 α2



D. DHD K12 β4 E. DHD K12 α6 F. DHD K12 α2



G. Negative H. Negative

Figure 6.1.

The α6 integrin (B&E) is stained with CY3 secondary (red). The other two integrins α2 and β4(A,C,D&F) are stained with FITC secondary antibody(green). Dapi is used to stain the nuclei blue. Expression of all these integrins is demonstrated by immunocytochemistry. The negative picture (H) and that at a lower magnification (G) show that no background fluorescence or non specific binding has occurred. In picture (D), the cells stained positive for the β4 integrin signal appear less in comparison with other two integrins in the DHD K12 cell line. This is artefactual due to cells overlying. The overlying cells cause the fluorescence signal obtained to arise from different depths and so diminish fluorescent intensity.

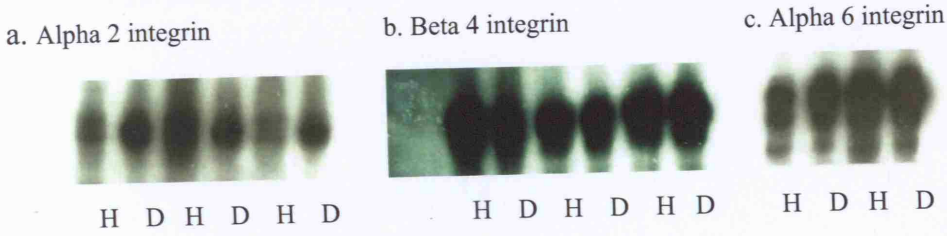
Immunocytochemistry shows that the three integrins are expressed strongly in the rat and human colorectal cancer cells. The negative control shows that no significant background fluorescence or non specific binding has occurred.

6.3.2 Western Blotting

Western Blotting was performed to quantify protein expression. Intense bands are demonstrated in both cell lines - α2 subunit at 165 kDa, α6 120 kDa and β4 220 kDa (Fig 6.2.).

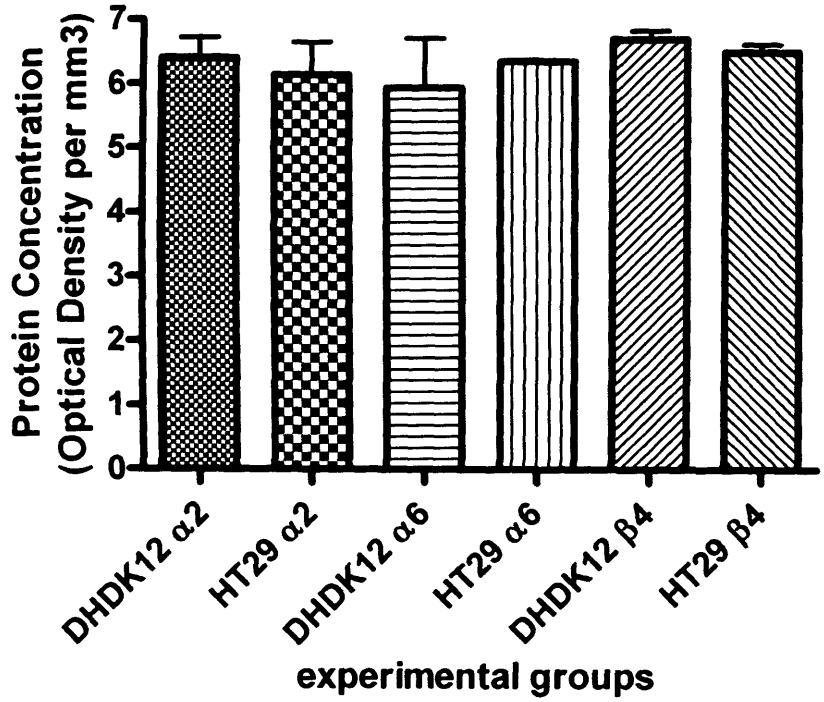
Analysis of these blots, by means of optical density, allowed more accurate quantification of protein concentration in the two cell lines. This is depicted graphically (Fig 6.3.). As each lane on the Western Blot was loaded with a uniform concentration of cell lysate, differences in protein expression have been observed both between and within the cell lines.

Figure 6.2.



Western Blots were performed on equal concentrates of cell lysate from both cell lines. Detection post incubation with the specific primary antibody, (α2 and β4 polyclonal antibody and α6 monoclonal) using chemiluminescent immunodetection was performed. (A) shows the bands obtained for alpha 2 integrin, (B) beta 4 and (C) alpha 6. In each analysis, HT29 (H) and DHD K12 (D) were run in alternative lanes.

Fig 6.3.



The graph above compares the protein concentrations of the different integrins examined. The protein concentration is calculated by measuring the density (optical density per mm^2) in each band and subtracting from this the mean background (optical density). The mean density for each integrin was then calculated and plotted on the graph. Similar integrins, from each cell line, were plotted next to each other.

The results support the findings that these three integrin subunits are conserved in different species of colorectal cancer and maintained expression occurs in phenotypically different HT29 cell lines.

6.4 Discussion

Integrin subunits α6, α2 and β4 are known to be expressed in human HT29 colorectal cancer (Haier *et al.*, 1999a) and play a role in extravasation (Enns *et al.*, 2004). In this study, using the techniques of Immunocytochemistry and Western Blotting, integrin expression in two unrelated cancer cell lines was compared. The results show that these integrins are expressed both in this phenotypically different HT29 cell line and in the rat colorectal cancer cell line DHDK12.

These results are significant. They not only demonstrate conservation of these three integrins in unrelated rat species but maintained expression of these integrins among the HT29 population. The previous characterisation of HT 29 (Haier *et al.*, 1999a) was conducted in1999 in the United States of America. Those HT29 cells were cultured in Dulbecco's modified Eagle's medium/F12. The analysed cells had undergone fewer than 15 passages (Haier *et al.*, 1999a). These HT29 cells used in the experiment, purchased from the European Collection of Cell Cultures (ECACC), are quoted as having undergone 135 passages before purchase. These HT29 cells, although having originated in the United States of America, are cultured in different media – McCoy's 5a. Despite the differences in cellular phenotype and passage number between the two HT29 cell lines, the α6, α2 and β4 integrins are maintained.

This finding emphasizes the functional importance of these molecules to the colorectal cancer cell lines. The integrins are tightly conserved molecules (Hynes, 2002). For colorectal cancer cell lines from different species to express the same integrin subunits suggests these molecules have an important role in cell function. In HT29 colorectal cancer cells, inhibition of these integrin subunits impaired tumour cell function (Enns *et*

al., 2004). Functional blocking of the α2 integrin subunit significantly inhibited tumour cell migration (Enns *et al.*, 2004). Functional blocking of the α6 and β4 integrin subunits impaired both tumour cell adhesion and migration (Enns *et al.*, 2004). *In vivo* work is required to establish the function of these integrins in the DHDK12 cell line. Creation of colorectal cancer cell mutants with alterations in integrin expression will assist in providing further insight into the roles of these molecules in the early stages of metastasis development. Mutants, with down regulation or up regulation of integrin expression, could be assessed by Western Blotting and protein quantification or by PCR.

With best conventional chemotherapy, median patient survival with metastatic colorectal cancer is 24 months (Kelly C and Cassidy J, 2007). The first principle of the modified "seed and soil" hypothesis states that cancers are a heterogeneous population of cells and each subpopulation has its own phenotype (Fidler, 2003). Conventional chemotherapy may be selecting out resistant cancer cells in patients – possibly cancer stem cells (Bao *et al.*, 2006;O'Brien *et al.*, 2007). This would account for the fact that patients initially can have a very positive response to treatment, followed by progression of local and metastatic disease. Altering chemotherapy regimes may temporarily hold disease progression in check. However for significant oncological advances to be made, alternative strategies for management of disseminated cancer must be deployed. The antiangiogenic therapies are hopefully just the start of this arduous process(Hurwitz *et al.*, 2004).

The integrin subunits α6, α2 and β4 could allow specific targeted therapies to be administered to colorectal cancer cells. Highly selective inhibitors could be used, not only to functionally block these molecules, but to deliver toxic anti-cancer therapies.

This would be more efficient and effective than conventional oncological therapies. Such treatment modalities would also significantly reduce the systemic effects that are currently associated with chemo and radiotherapy – effects that can be so severe as to cease treatment tolerance. The specificity of such a system would increase if integral components of the downstream pathways, activated by the integrins, could be identified and targeted as the integrins are expressed in a variety of human cells.

Experiments to ascertain which healthy cells express these integrins would need to be performed. Antibodies, specific to the α2, α6 and β4 integrin subunits, could be tagged with a non harmful label and introduced into a rodent host to see where binding occurs. This would identify the tissues and organs where local side effects would occur. The systemic manifestations of this novel therapy could then be calculated.

In conclusion, the expression of α6, α2 and β4 integrins in phenotypically different HT29 cells and in unrelated rat colorectal cancer suggests these molecules have an important function. These molecules may allow therapeutic interventions in the early stages of colorectal cancer liver metastasis development. They may also enable targeted therapies to be administered specifically to colorectal cancer cells.

CHAPTER 7

Functional Blocking of Specific Integrins Inhibits Colonic Cancer Cell Migration

7.1 Introduction

Recent research has focused on methods to increase the clinical management of local and disseminated colorectal carcinoma. Conventional chemotherapy, despite advances (Allegra and Sargent, 2005;Twelves *et al.*, 2005), can only give patients with disseminated disease a median life expectancy of two years (Kelly C and Cassidy J, 2007). The liver is the most common and critical site of distant metastasis. At diagnosis, as many as 25% of colorectal cancer patients have established liver metastases (Kavolius *et al.*, 1996). In advanced disease, these metastases destroy the anatomical architecture of the liver, impairing hepatic function leading to the demise of the patient.

As early as 1889, Stephen Paget's "seed and soil" hypothesis proposed that metastasis occurred in a non random pattern. (Paget S., 1889). The "seed and soil" hypothesis has been adapted and the modified hypothesis consists of three separate entities to propose how metastasis develops (Onn and Fidler, 2002;Fidler, 2003). These principles provide us with insights into how advances in the management of disseminated disease can be achieved. Firstly, cancers consist of different cell subpopulations each of which has its own phenotypes. Current research (Bao *et al.*, 2006;O'Brien *et al.*, 2007) is closely examining these cell subpopulations to determine whether every cancer cell possesses the ability to initiate and sustain tumour growth or whether only a subset of cells, cancer stem cells, possess such potential. Recent work (O'Brien *et al.*, 2007) involving human colon cancer-initiating cells in immunodeficient mice showed that all human colon cancer-initiating cells were CD133+. The majority of the tumour cells were CD133- and were unable to initiate tumour growth. In glioma tumours, CD133+ cells were also identified (Bao *et al.*, 2006). Within glioma tumours *in vitro* and *in vivo*, a higher proportion of these CD133+ cells were found to survive ionising radiation. These

CD133+ cells were found to initiate DNA repair, post radiotherapy, more effectively than CD133- cells. These results suggested that the CD133+ cells confer radioresistance on glioma tumours and could cause tumour recurrence post radiation therapy. These results suggest that a cancer hierarchy may exist. Only a proportion of tumour cells may be responsible for tumour proliferation. These stem cells may also be responsible for tumour recurrence after conventional oncological management. Significant advances in oncological management may occur through targeted cytotoxic therapies directed at these cancer stem cells.

The third principle states metastasis requires multiple interactions between the tumour cell and the regulatory mechanisms of the adjacent microenvironment (Liotta and Kohn, 2001;Fidler, 2002). The clinical application of this principle has led to therapies disrupting the tumour cell-microenvironment interaction. Novel therapies have been devised to interfere with the neo-angiogenesis process. Bevacizumab, a selective inhibitor of Vascular Endothelial Growth Factor (VEGF), has been used as an effective adjuvant to standard chemotherapy for metastatic colorectal cancer(Hurwitz *et al.*, 2004).

The second principle states that the process of metastasis is selective for tumour cells which have the ability to embolise, invade, adhere, extravasate and establish metastasis in distant organs. Recent research suggest that the integrins play a key role in many of the above processes (Enns *et al.*, 2004;Jin and Varner, 2004;Enns *et al.*, 2005;Eble and Haier, 2006;Tucker, 2006). This paper examines the key roles that specific integrins play in colonic cancer migration and how these molecules may enable more specific anti-cancer therapies to be developed.

Colorectal cancer cell migration from the hepatic sinusoids into the liver parenchyma is a crucial step in the development of liver metastasis (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). It is believed to swiftly follow colorectal cancer cell targeting and adhesion (Haier *et al.*, 2003;Enns *et al.*, 2004;Gassmann *et al.*, 2004;Enns *et al.*, 2005). Successful extravasation confers many advantages on the tumour cell. Escaping the circulation protects the cancer cells from haemodynamic stresses, which can rapidly kill tumour cells (Brooks, 1984). As well as obtaining nutrients within the liver parenchyma, certain colorectal cancer cells can interact with local regulatory mechanisms to stimulate self-proliferation and neo-angiogenesis (Chambers *et al.*, 2002;Fidler, 2002).

Successful extravasation requires the completion of multiple steps. The tumour cell must possess not only the ability to adhere to the target organ but to modify and renew these adhesions in a dynamic fashion (Von *et al.*, 2005). The tumour cell may then advance through degradation of the local extracellular matrix. The cell must also have the ability to avoid apoptosis and anoikis. Anoikis is the induction of apoptosis due to the lack of cell binding to other cells or to a basement membrane.

Recent work by Enns et al (Enns *et al.*, 2004) has shown that functional blocking of integrin subunits α6, α2 and β4 resulted in significant inhibition *in vivo* of human HT 29 colorectal cell migration. The exact mechanism is unknown. The principal focus of their work was analysis of HT29 colorectal cancer cell adhesion within the sinusoids and the integrins involved. The number of colorectal cancer cells, which extravasated during their half an hour observation period, was small. The biological accuracy of their model – using human cancer cells in a rodent host – has to be questioned. Despite statistical analysis of human HT29 and rat CC531 colorectal cancer cell lines (Haier *et al.*, 2003)

revealing no differences over the duration of the experiment, differences between the species in hepatic architecture and homeostatic regulation are likely to influence and distort metastasis development.

Having established both the rat DHDK12 and the phenotypically different human HT29 colorectal cancer cells lines express the three integrin subunits - α6, α2 and β4, the role of these integrins was examined *in vivo*. These three integrins are known to be involved in cell migration in the human HT29 cell line(Enns *et al.*, 2004). The *in vivo* experiments specifically focussed on the role of these subunits in the DHDK12 cell line.

7.2. Methods and Materials

7.2.1 Cell Culturing, Labelling and Function Blocking

As described in 4.2.1 and 4.2.2. For experiments involving integrin functional blocking, 1:150 concentration of antibody was added to the cell-Calcein AM-suspension one hour prior to animal introduction and incubated in humidified 5% CO2/ 95% air at 37°C.

7.2.2 Animals

As described in 4.5.

7.2.3 Statistical analysis

As described in 4.6.

7.3 Results

7.3.1 The Early Events of Metastasis Development – Experimental Findings

Five experimental groups (n=6) – DHDK12 wild type (WT DHDK12), DHDK12 and non specific IgG (IgG DHDK12), DHDK12 and alpha 2 polyclonal antibody (α2 DHDK12), DHDK12 and beta 4 polyclonal antibody (β4 DHDK12) and DHDK12 and alpha 6 monoclonal antibody (α6 DHDK12) – and a sham group (n=3) were compared.

7.3.2 Cells Migration Rate and Max Migration Rate

Cell migration rates were shown in Fig 7.1. Cell migration rates increased during the experimental period in all 5 groups. Statistical analysis of cell migration showed that the migration rates of WT DHDK12 and IgG DHDK12 groups were significantly higher than the other three groups, where specific integrin subunits had been functionally blocked ($p<0.001$) (Fig. 7.2.). A comparison of the maximum migration rate between the groups, defined as the highest migration rate recorded in each experiment, showed significantly higher ($p<0.001$) values in the WT DHDK12 and IgG DHDK12 groups (Fig 7.3). For cell migration and the maximum cell migration there was no significant difference detected between the α2 DHDK12, β4 DHDK12 and α6 DHDK12 groups or between the WT DHDK12 and IgG DHDK12 groups

7.3.3 Extravasated Cells

The extravasation of cancer cells in the five experimental groups were analysed (Fig 7.4). The number of extravasated cells increased in all five groups over the two hour observation period. In Fig 7.4, this increase and the number of extravasated cells visualised was more marked in the WT DHDK12 and IgG DHDK12 groups. Although analysis of the area under the graph (Fig 7.5) found a significant difference between the

means for the groups in a one way variance of analysis ($p<0.0028$), with Bonferroni's Multiple Comparison Test no significant differences between the groups were evident.

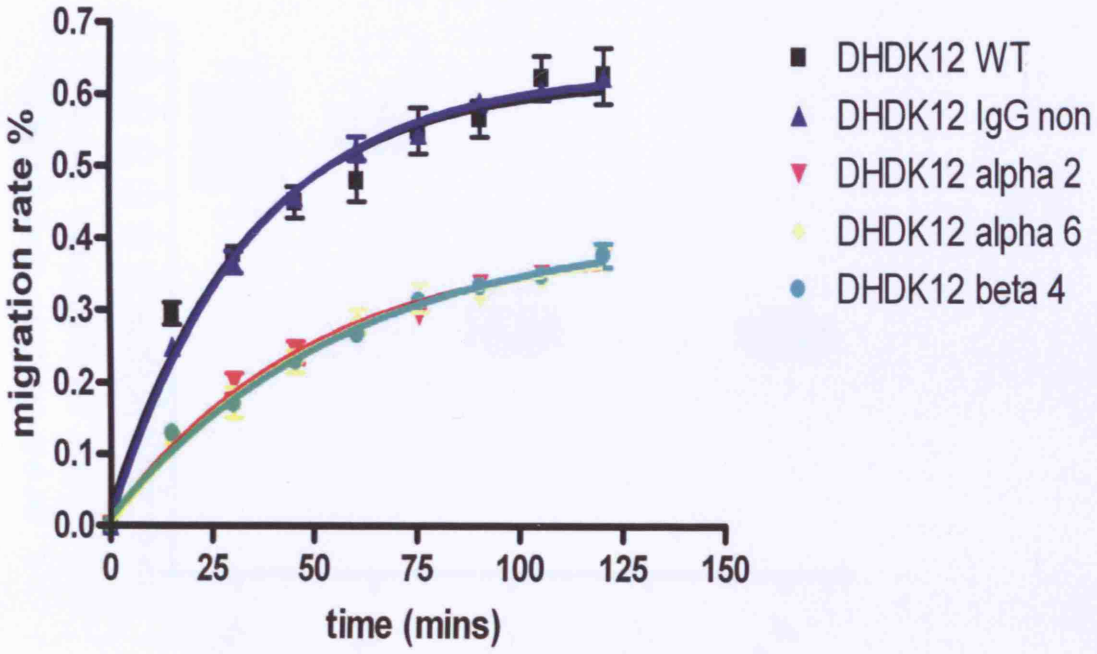


Fig 7.1.
This graph shows the migration rates for the five experimental groups throughout the two hour experimental period. The migration rates are similar for the WT and WT with Non Specific IgG. The migration rates are significantly less for the other three groups where integrin subunits α2, β4 and α6 have been blocked with antibodies. The migration rates are very similar for these three groups.

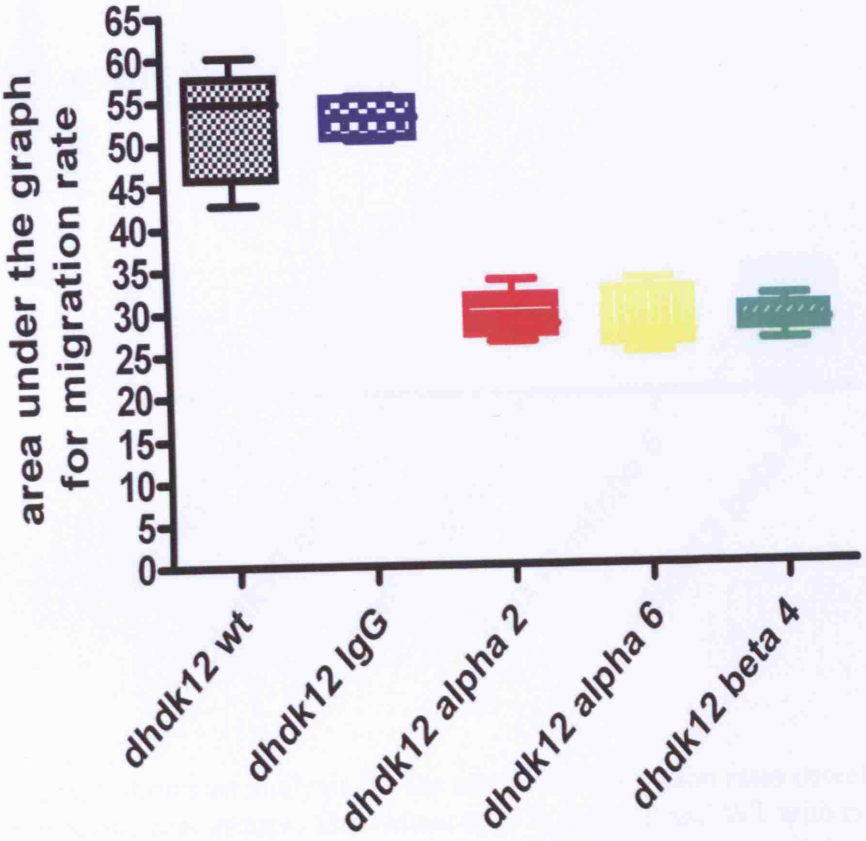


Fig 7.2.
This graph shows an analysis of the migration rates for the different groups. When statistical comparisons are made between the groups, the WT and WT with non specific IgG are statistically higher ($P<0.001$).

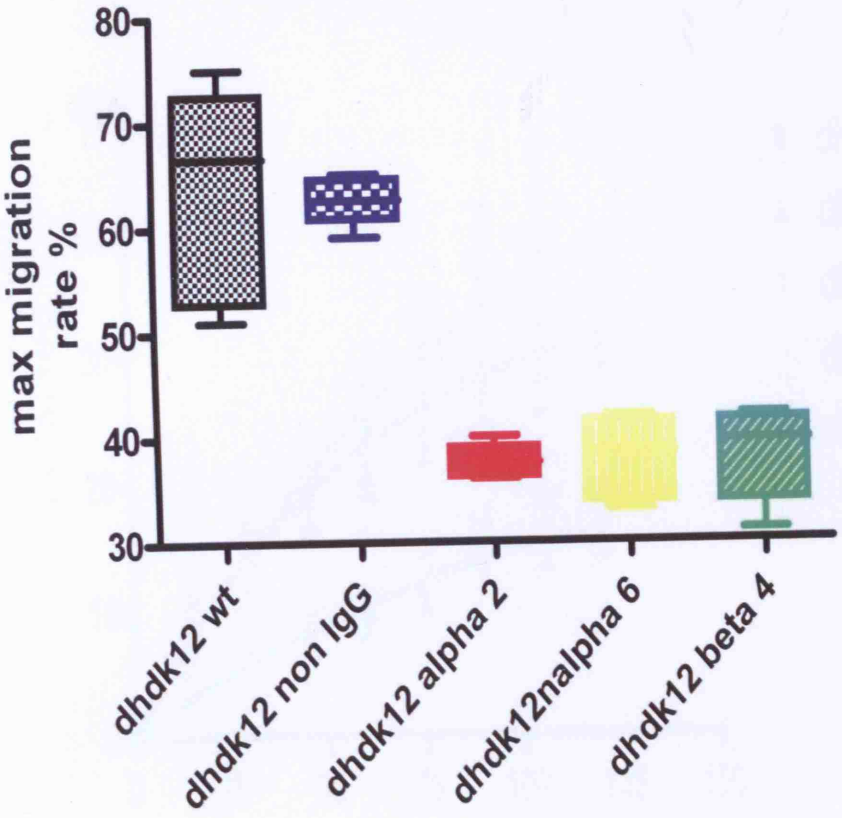


Fig 7.3.
This graph shows an analysis for the maximum migration rates detected in each of the five experimental groups. The values seen in the WT and WT with non specific IgG are statistically higher ($p<0.001$)

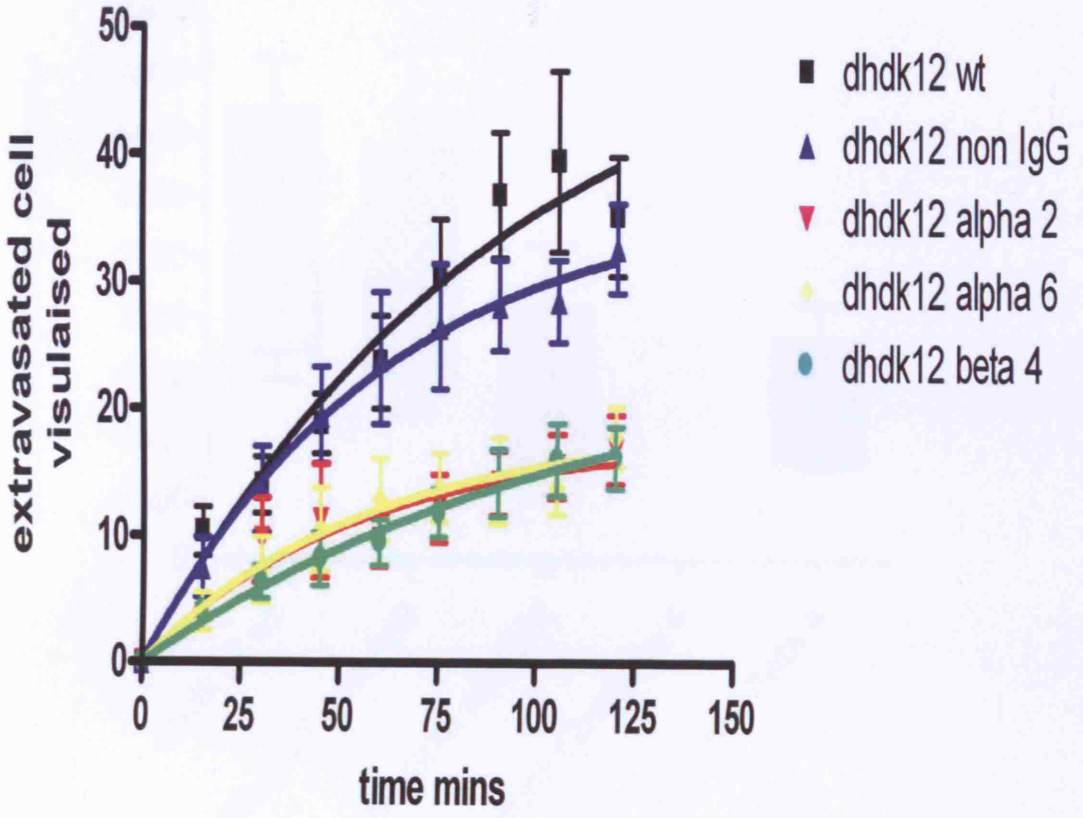


Fig 7.4.
This graph plots the number of extravasated tumour cells visualised during the experiment for the different groups.

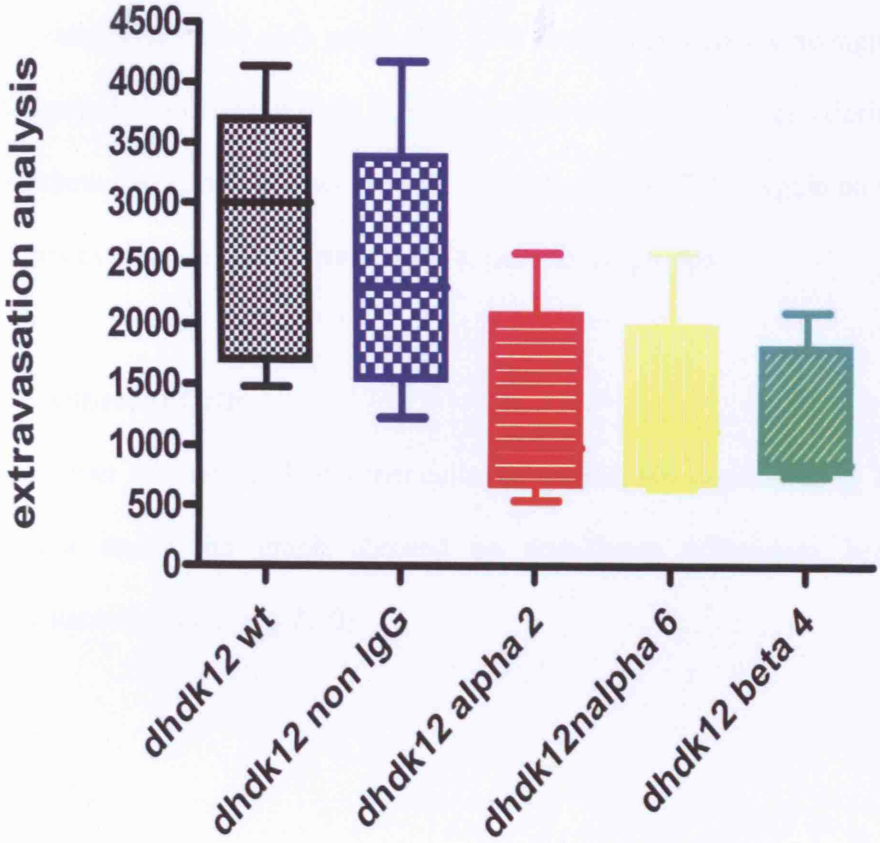


Fig 7.5.
This graph shows an analysis for the number of extravasated cells visualised. Statistical analysis was performed. ANOVA one way analysis reveals a significant difference between the group means (p 0.0028). Bonferroni's Multiple Comparison Test however reveals no significant differences between the groups.

7.3.4 Total number of visualised cancer cells

The total number of visualised tumour cells throughout the experiments for each of the five groups is shown in Fig 7.6. No obvious differences are evident between the groups. Analysis of area under each graph (Fig 7.7.) shows that there are no significant differences between the groups. Analysis of the maximal cells seen during the experiment was compared between the five groups (Fig 7.8.). Again no significant differences were detected between the experimental groups.

7.3.5. Adherent Cells

The number of visualised adherent cells are graphically depicted (Fig 7.9). Analysis of the area under the graph showed no significant differences between the five experimental groups (Fig 7.10).

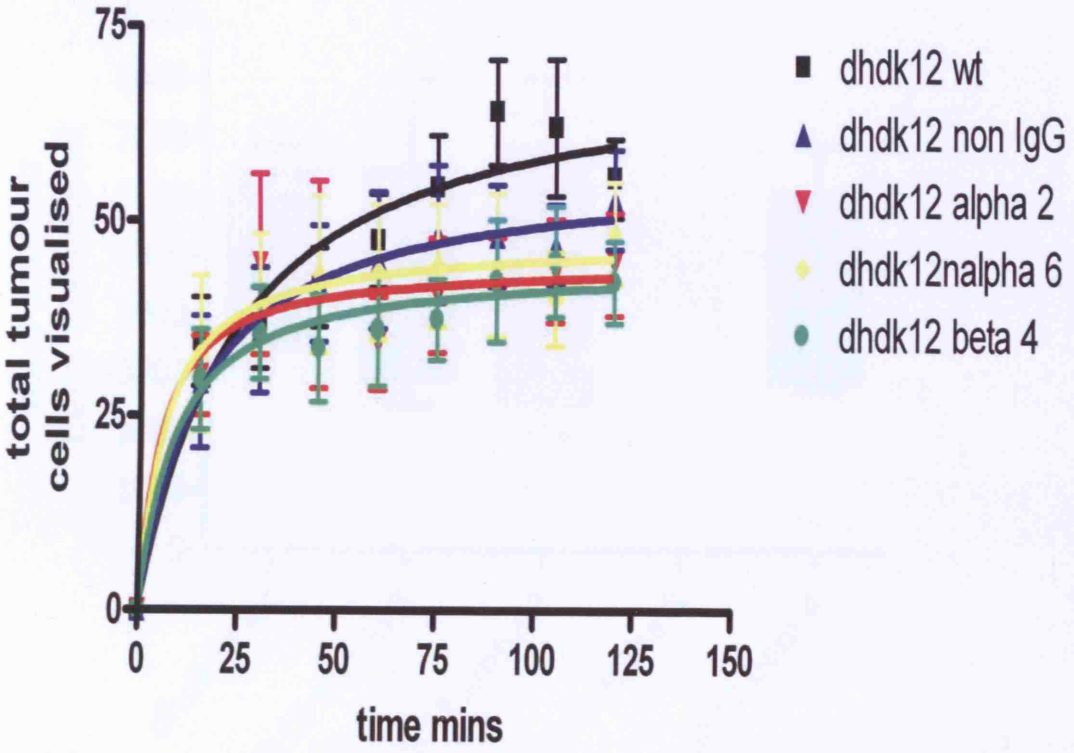


Fig 7.6.
This graph shows the total number of tumour cells visualised in each of the five experimental groups throughout the experiment.

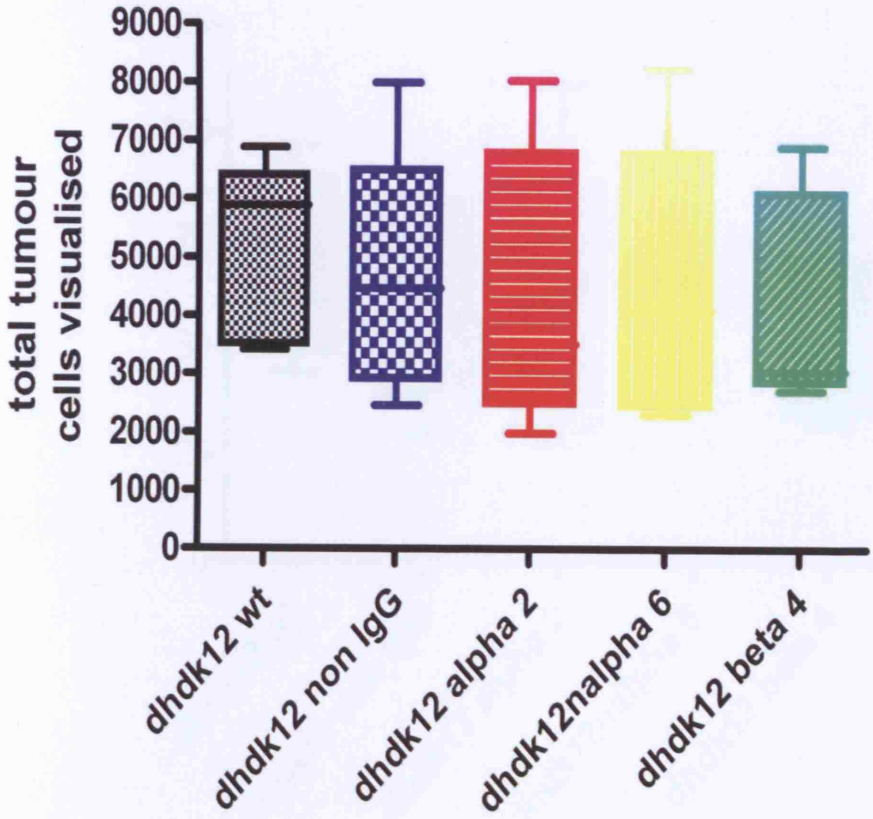


Fig 7.7
This graphs shows the analysis of the area under the graph for each of the experimental groups. The total number of tumour cells visualised is compared. No significant differences were detected.

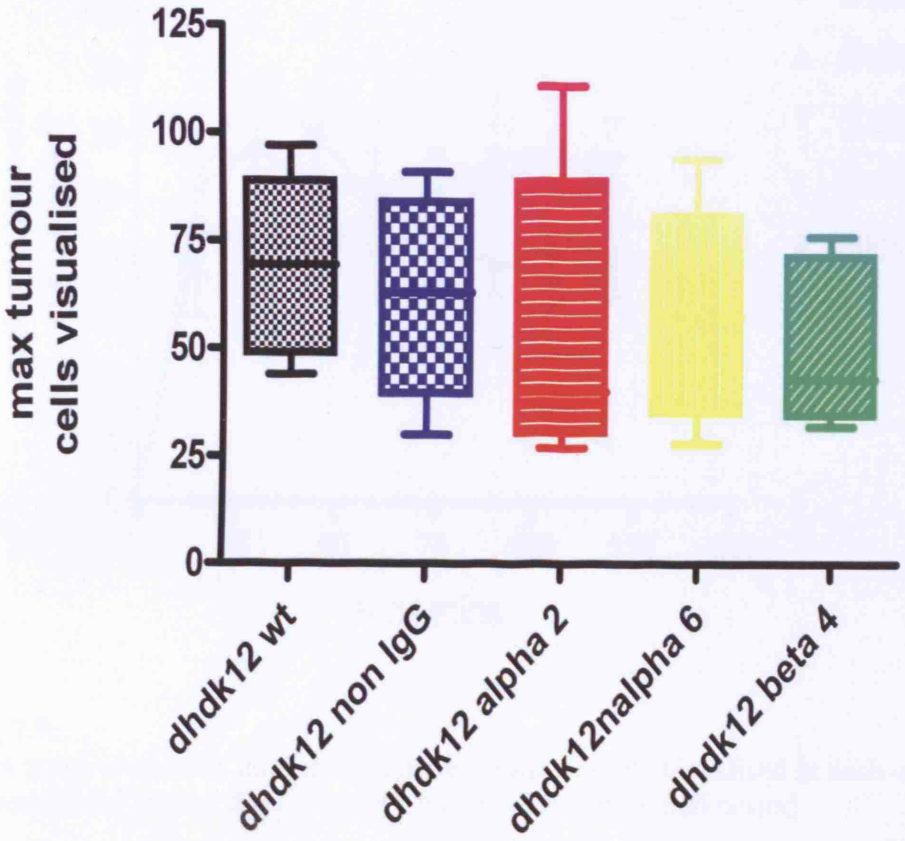


Fig 7.8
This graph shows the analysis of the maximum tumour cells visualised between the experimental groups. No significant differences were detected.

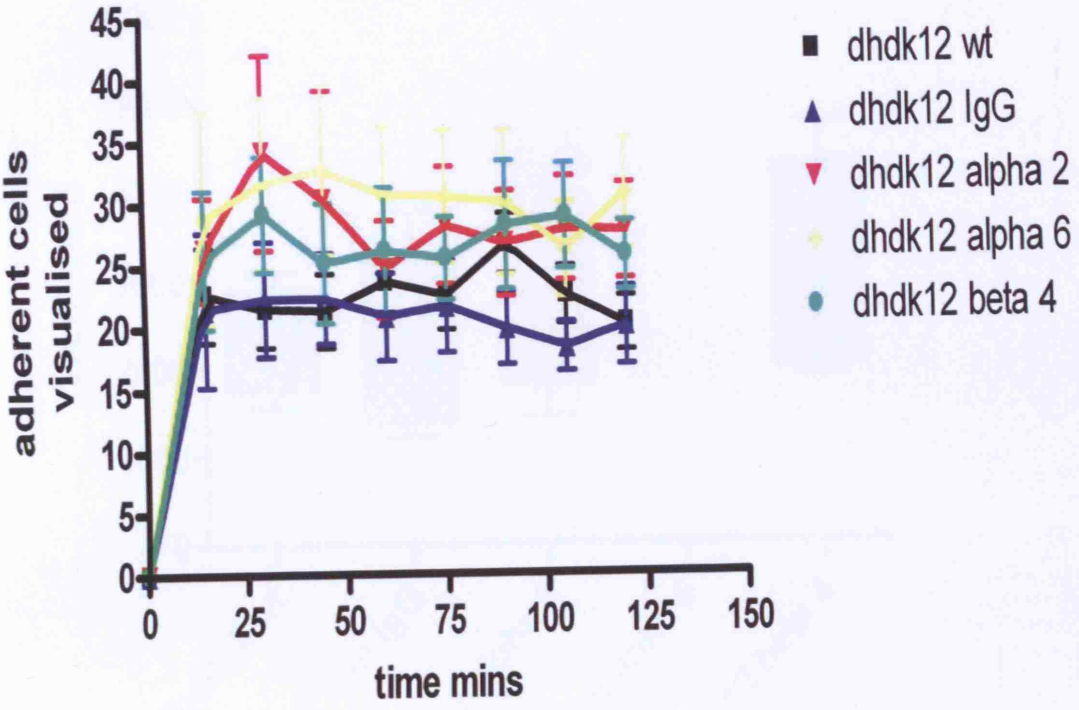


Fig 7.9.
This graph shows the number of adherent tumour cells visualised in each of the experimental groups throughout the two hour experimental period

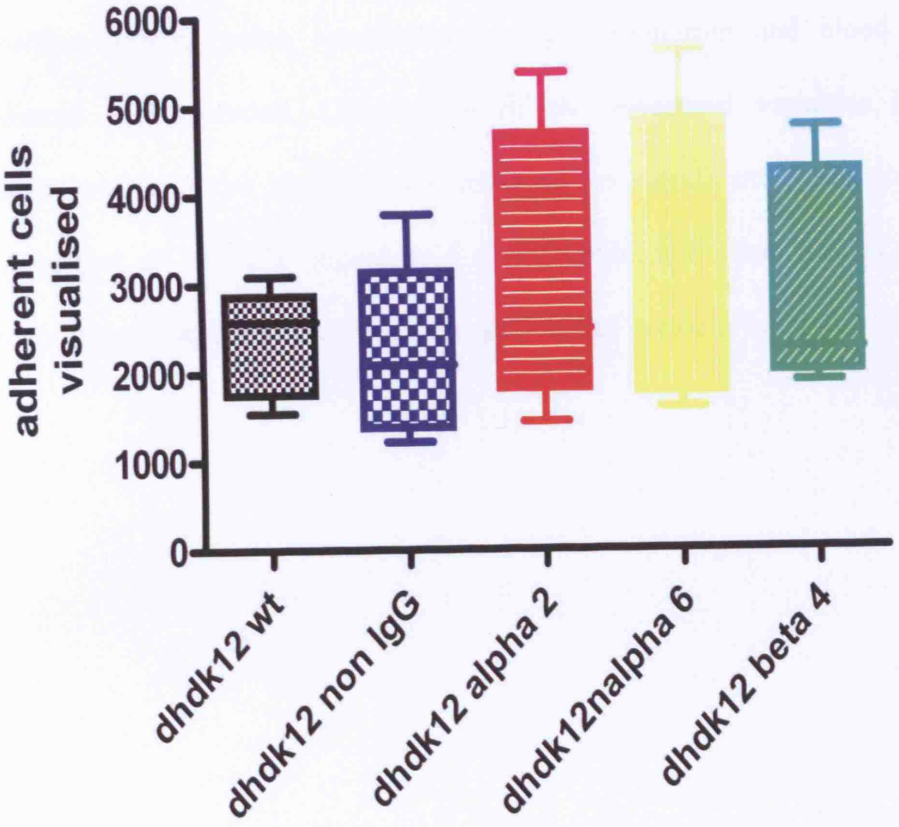


Fig 7.10
This graph shows an analysis of the number of adherent tumour cells visualised. No significant differences were detected.

7.3.6 Systemic Haemodynamic Variables

To study the effect of the experimental procedures, particularly the 1.5 ml intra-arterial injection volume, on the systemic variables, a sham group (n=3) was included. The sham operation was the exact same as the standard procedure, but an intra arterial injection of PBS was introduced instead of the cancer cell suspension. Animal systemic variables such as pulse, temperature, oxygen saturation and blood pressure were measured and recorded. Comparison of the measured variables between the 5 experimental and the sham groups revealed no significant differences. The results showed that all animals (including 5 experimental and sham groups - 33 rats) were physiologically stable throughout the experimental period (Fig 7.11-7.14).

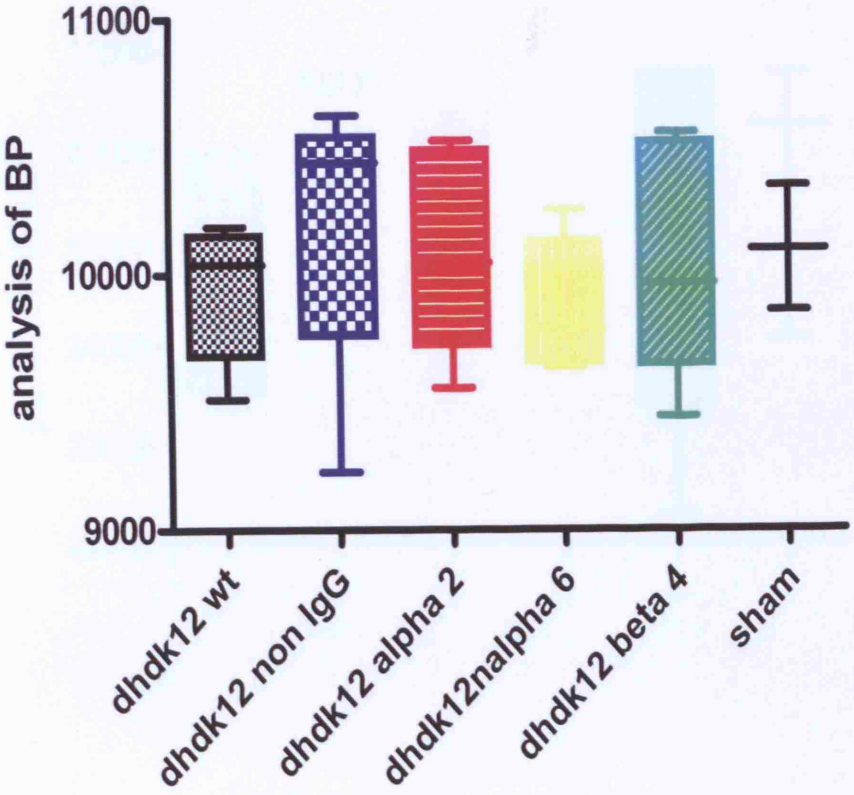


Fig 7.11
This graph show a comparison of the blood pressures detected during the experiments for the six groups. There are no significant differences between the experimental groups and the sham experiment.

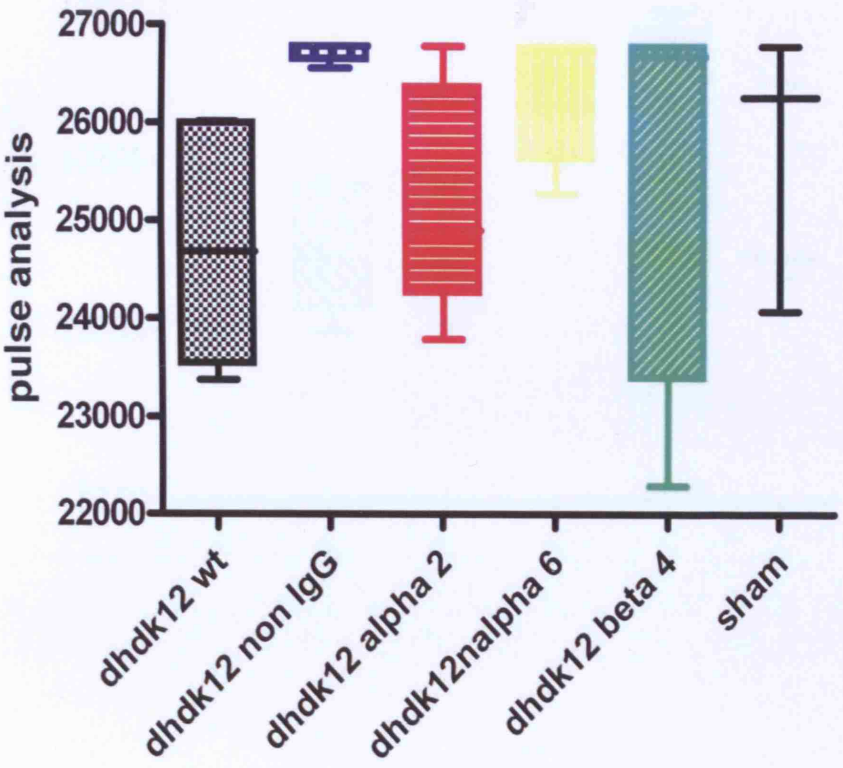


Fig 7.12
This graph show a comparison of the heart rates detected during the experiments for the six groups. There are no significant differences between the experimental groups and the sham experiment.

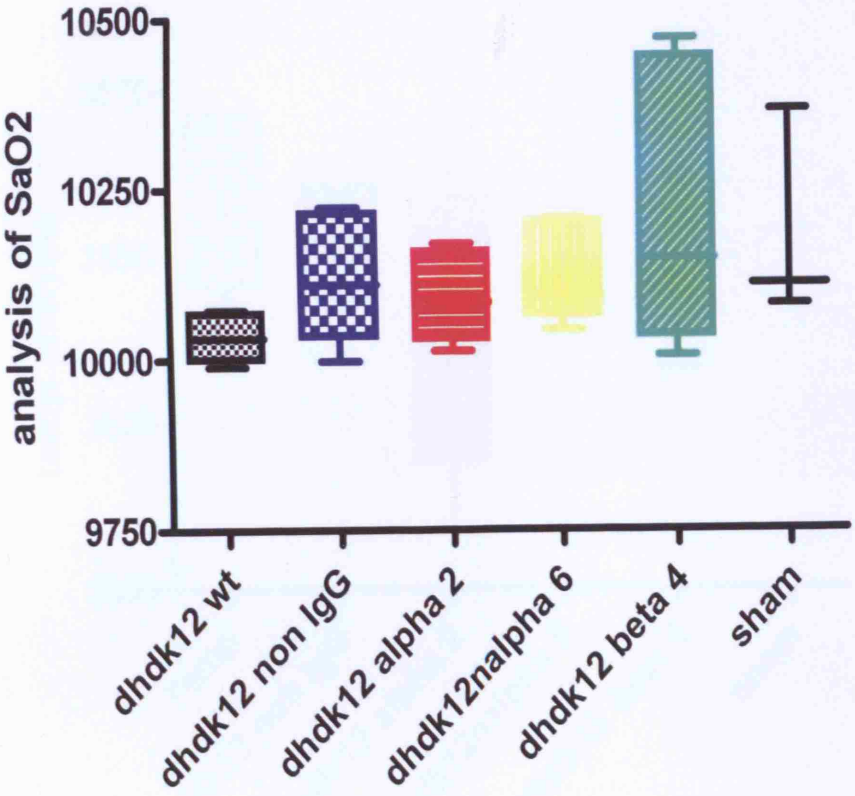


Fig 7.13
This graph show a comparison of the oxygen saturations detected during the experiments for the six groups. There are no significant differences between the experimental groups and the sham experiment.

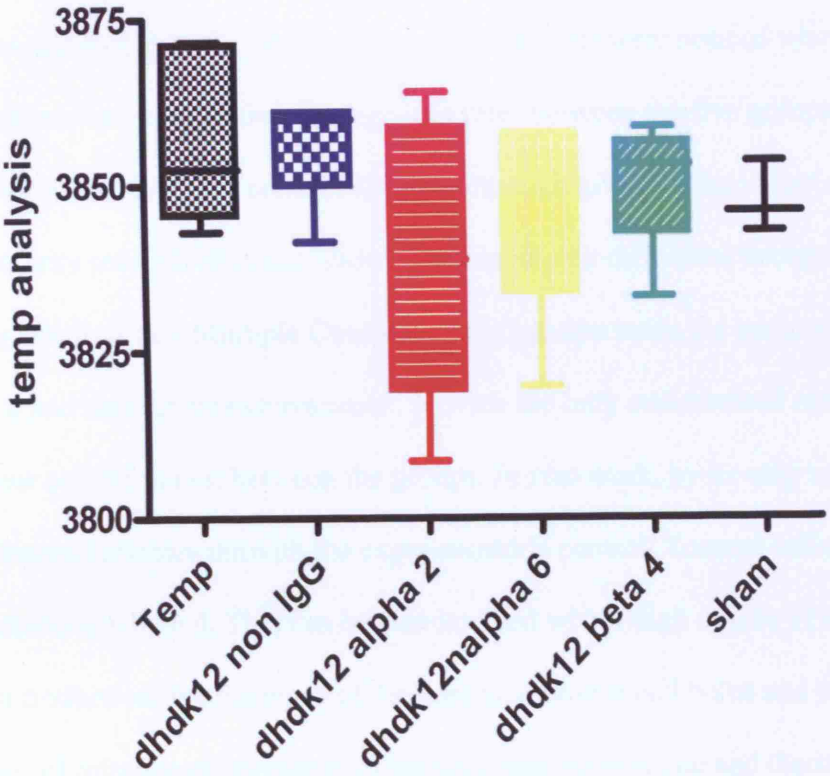


Fig 7.14
This graph show a comparison of the temperatures recorded during the experiments for the six groups. There are no significant differences between the experimental groups and the sham experiment.

7.4. Discussion

These results suggest that these three integrin subunits - α6, α2 and β4 – play a key role in rat DHDK12 colonic cancer cell migration.

In this study, significant differences in all statistical variables (one way analysis of means and Bonferroni's Multiple Comparison Test) were noticed when comparing only migration rates and maximum migration rates between the five groups. Analysis of the number of extravasated cells visualised, although giving a significant result with regard to one way analysis of mean, showed no significant difference between the groups when using Bonferroni's Multiple Comparison. Migration rates, the percentage of total cells which had undergone extravasation, provide the only standardised method to compare tumour cell migration between the groups. *In vivo* work, by its very nature, has a high number of variables outwith the experimenter's control. Tumour cells must be cultured, counted and labelled. This can be standardised with a high degree of accuracy. Tumour cell introduction, the response of the host to a tumour cell bolus and optimisation of images of intrahepatic events with the intravital microscope and thereafter accurate analysis of the data introduce further variables. The total number of tumour cells visualised varied not only between the different experimental groups but in experiments within the same groups. Migration rate, therefore, provides a standardised and most accurate method to compare extravasation rates between the groups.

Analytical comparisons of the wild type DHDK12 and the non specific IgG DHDK12 groups show very similar migration rates. Functional blocking of specific integrin subunits α6, α2 and β4, although producing different total tumour cells, adherent and extravasated cells visualised, produce very similar migration rates. This inhibition of migration is consistent with Haier et al results (Enns *et al.*, 2004). The very similar

migration rates could be attributed to activation of a common downstream pathway or direct interference of the tumour cell antibody complex with the migration process. These results suggest that the colonic cancer cell antibody has no significant effect on tumour cell adhesion. In the work by Haier et al (Enns *et al.*, 2005), antibody inhibition of the αv integrin subunits significantly reduced metastatic cell adhesion. They report that, although absolute numbers of extravasated tumour cells were reduced – secondary to impaired tumour cell adhesion, the relative migration rates were not affected by αv integrin blockade. To further analyse and clarify the roles of the integrin subunits α6, α2 and β4 in tumour cell migration and disprove the theory of direct antibody interference, knockout mutant models are required. Comparisons of wild type, knockout mutants and mutants with up-regulated expression of a specific integrin sub-unit would help to further clarify the mechanism of action. Future work could also examine the effect of simultaneously blocking several integrins, known to be involved in colonic cancer cell migration, to see if there is a synergistic effect.

This *in vivo* work on early colonic cancer hepatic metastasis development supports the findings of Haier et al with regards to the progression of the early stages (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006). Intra-arterially introduced colonic cancer cells were found to adhere to the sinusoid vessel walls in patent vessels – supporting the theories of tumour cell targeting and cell specific adhesion. Over the duration of the experiments, the cells were noted to extravasate. No evidence of intra-vascular proliferation was seen (Al-Mehdi *et al.*, 2000).

The role of integrins in specific tumour cell adhesion is well understood. The role of integrin subunits in tumour cell migration is less clear. At least three distinct

mechanisms of action have been proposed – direct; modification of tumour cell phenotype or enzymatic recruitment and regulation of expression.

A direct mechanism of action has been identified in endothelial cells where the integrin αvβ3 is specifically recruited to the leading edge of lamellipodia during migration (Kiosses *et al.*, 2001). Alternatively the integrins may induce alteration of cellular phenotypes. In prostate cancer cells (Zheng *et al.*, 2000), the αvβ3 integrin has been shown not only to change affinity for ligands in different functional states but also activate cell migration by different routes depending on different ligand binding. The αvβ3 integrin was shown to both activate the phosphatidylinositol 3- kinase/ protein kinase B pathway and PI 3-kinase to induce migratory characteristics (Zheng *et al.*, 2000). The αvβ3 integrin, again in prostate cancer cells (Manes *et al.*, 2003), has been shown to alter cdc 2 protein and kinase activity levels. Raised cdc 2 levels were found to increase cell motility - the cdc 2 protein being the downstream effector of the αvβ3 integrin. The αvβ3 integrin transmits signals from the external environment into the cell, modifying the cellular characteristics.

In vitro work in both human dermal microvascular endothelial cells (HMEC) and human breast tumour-derived endothelial cells (B-TEC) further emphasises the importance of metalloproteinases and integrins. Oxytocin in these cells was found to induce proliferation and migration. A comparison of treated and non-treated cells revealed oxytocin had caused specific matrix metalloproteinases and the integrin β6 gene to be overexpressed (Cassoni *et al.*, 2006). This alteration of migratory characteristics is vital during extravasation. The integrins, while stimulating these changes (Zheng *et al.*, 2000;Manes *et al.*, 2003), participate in proteolytic enzyme recruitment and targeting. Recent work in breast cancer (Rolli *et al.*, 2003) has shown a

relationship between expression of activated αvβ3 and the production of active MMP-9. In colorectal cancer, the enzyme gelatinase has been identified and this may assist with the metastatic colonisation of the liver (Tien *et al.*, 2003). Directing these enzymes to the appropriate cellular location can also be attributed to the integrins. The proteolytically active form of MMP-2 binds directly with αvβ3 in angiogenic blood vessels and melanoma cells *in vivo* (Brooks *et al.*, 1996).

The significant inhibition of colonic cancer migration has implications for future oncological therapies. 25% of newly diagnosed colorectal cancer patients have established liver metastases. If these integrin subunits are important for extravasation in a wide variety of colorectal cancer cell strains, functional blocking of these integrins in the human patient would be advantageous. Inhibition of extravasation would impede tumour cell escape from the circulation. The cancer cells would have prolonged exposure to physiological shear stress which would prove lethal to many (Brooks, 1984). Inhibition of extravasation would inhibit new metastasis development in patients with established metastasis. More aggressive local oncological management, through surgery or radiofrequency ablation, could be routinely considered.

These integrin subunits α6, α2 and β4 could allow specific targeted therapies to be administered to colorectal cancer cells. Highly selective inhibitors could be used, not only to functionally block these molecules, but to deliver toxic anti-cancer therapies. This would be a more efficient and effective than conventional oncological therapies. Such treatment modalities would significantly reduce the systemic effects that are currently associated with chemo and radiotherapy – effects that can be so severe as to cease treatment tolerance. The specificity of such a system would increase if integral

components of the downstream pathways, activated by the integrins, could be identified and targeted as the integrins are expressed in a variety of human cells.

Finally, Haier et al (Enns *et al.*, 2004;Enns *et al.*, 2005) showed that, despite blocking multiple cell adhesion molecules, complete inhibition of adhesion was never achieved. Similar findings are likely to be true with regard to extravasation. Many different molecules are likely to be involved in colorectal cancer cell migration.

In conclusion, integrin subunits α6, α2 and β4 have a role in tumour cell migration in the rat DHDK12 colonic cell line.

CHAPTER 8

General Discussion,

Future Work & Conclusions

8.1 Discussion

The primary locus of the colorectal tumour can frequently be managed by surgery either alone or in conjunction with neoadjuvant and adjuvant oncological therapy (Nelson *et al.*, 2001). Management of the metastatic spread is more difficult. After surgical resection, patients face a 40-50% chance of relapse (Obrand and Gordon, 1997).

The liver is the most common and critical site of distant metastasis. Despite advances in the oncological management of disseminated colorectal cancer (Allegra and Sargent, 2005;Twelves *et al.*, 2005), surgical resection of liver metastases remains the only therapeutic intervention that offers the possibility of long term survival and cure (Mutsaerts *et al.*, 2005). Chemotherapy regimes have advanced from 5 Fluorouracil (5FU) and Folinic Acid to regimes involving combinations of 5FU with oxaliplatin and irinotecan. Despite improvements in patient survival with these more modern chemotherapy regimes, median patient survival with metastatic colorectal cancer is 24 months (Kelly C and Cassidy J, 2007). For further advances to occur, in treatment of disseminated colorectal cancer, the key cellular molecules and processes involved in metastasis establishment must be identified. Increased understanding and identification of these processes will enable novel oncological therapies to be devised - more efficient and specific than conventional treatments. These novel therapies can then be used in combination with conventional chemotherapies to improve patient quality of life, disease free survival and life expectancy. Improved patient survival has already been seen when novel anti-angiogenic therapies, Bevacizumab, are combined with conventional chemotherapy in the treatment of metastatic colorectal cancer (Hurwitz *et al.*, 2004). Targeting of specific integrins may provide the answer.

8.2 Biologically Accurate In Vivo Models

To achieve this fuller understanding of the metastasis process, accurate *in vivo* models are required as was examined in Chapter 5 - *A New In Vivo Model for Early Development of Colorectal Liver Metastasis*. Creating an accurate biological model of metastasis is difficult. As discussed, many factors influence metastasis development. One of the key factors in achieving this biologically accurate model is cancer cell line–host combination. The results showed that this had a significant effect. In the novel highly biologically accurate model – DHDK12 in BDIX rat – significantly higher results were seen in all experimental variables. These results are further supported by work involving orthotopic models. Human colorectal cancer liver metastasis development was examined in female BALB/c mice (Flatmark *et al.*, 2004). 12 colorectal cancer cell lines were implanted and showed considerable variation with regard to tumour propagation and dissemination. Only one, of the twelve colorectal cancer cell lines, produced liver metastases. This occurred in only two of ten animals. In the orthotopic models, at least some of this variation was due to immunosurveillance and clearance. This theory was supported by further work looking at HT29 human colorectal cancer cells in mice. In immunocompetent mice, similar results were achieved to above (Flatmark *et al.*, 2004). However in the immunocompromised SCID mouse, significantly higher rates of lymph-node metastases and liver and lung metastases occurred (Guilbaud *et al.*, 2001).

Other factors, apart from the immune system, will influence metastasis development. It is important to return to the modified "seed and soil" hypothesis and especially, the second and third principles (Onn and Fidler, 2002;Fidler, 2003). The second principle states - the metastatic process is selective for tumour cells which have the ability to

embolise, invade, adhere, extravasate and establish metastasis in distant organs. The third principle states - metastasis requires multiple interactions between the tumour cell and the regulatory mechanisms of the adjacent microenvironment (Liotta and Kohn, 2001;Fidler, 2002). Current models use a variety of different cancer cell line-host combinations. Some groups have used syngenic combinations (Sturm *et al.*, 2003;Reinmuth *et al.*, 2003) while other research have used human cells in a rodent host (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005;Schluter *et al.*, 2006). Distantly related species and even some closely related species have significant anatomical and biological differences. These differences are likely to influence *in vivo* models of metastasis. In early models studying the development of colorectal cancer liver metastases, these differences are likely to make the metastasis process artificial and less efficient. For instance, the interaction of human colorectal cancer cells with a rodent liver (Haier *et al.*, 2003), a vital process in the conventional theory of cancer dissemination (third principle of modified "seed and soil" hypothesis), is at best a representation of hepatic colorectal cancer metastasis development in a human patient. This representation may, at times, be frankly misleading and is likely to underestimate the efficiency of colorectal cancer dissemination in the human patient.

This theory is supported by the results in Chapter 5. The DHDK12 colonic cancer cell line was chemically induced in the BDIX strain of rat (Martin *et al.*, 1983). Therefore the *in vivo* model of metastasis, DHDK12 cell line in the BDIX rat, is highly biologically compatible. Comparison of summary results of this group, with the other three experimental groups, showed a significant increase in every measured experimental parameter of metastasis development - total cancer cells visualised within the hepatic microcirculation, cancer cells adhering to the sinusoids, cancer cells extravasating, migration rate, maximum migration rate and maximum total cancer cells

visualised. No significant differences were noted between the three other groups. No significant differences in haemodynamic variables were noted between the two rat strains in the sham experiments. No significant haemodynamic variables were evident between the four experimental groups. These results demonstrate that cancer cell line-host selection and compatibility has significant effects on early metastasis development. Previous work (Chambers *et al.*, 2001;Chambers *et al.*, 2002;Onn and Fidler, 2002;Fidler, 2002;Fidler, 2003) has highlighted the importance of tumour cell interaction with both the organ of metastasis and with local host homeostatic mechanisms. This work shows not only does non-related cancer cell line-host combination affect metastasis development but the introduction of colonic cancer from a different strain within a species alters metastasis development.

This work, therefore, suggests that *in vivo* models, especially those studying the early stages of colorectal cancer liver metastasis development, should use a highly biologically compatible cancer cell line-host combination to achieve the most accurate picture of metastasis development possible.

8.3 Integrins: Future Therapeutic Targets?

The importance of the understanding of metastasis development is emphasised by the creation of new antiangiogenic therapies. These are now being used in combination with conventional chemotherapy to improve patient survival in metastatic colorectal cancer (Hurwitz *et al.*, 2004). In 1971, Folkman (Folkman, 1971) discussed the therapeutic implications of tumour angiogenesis. Research has led to increased understanding of the tumour angiogenesis process and the key role of Vascular Endothelial Growth Factor (VEGF). Subsequently, VEGF inhibitors have been developed and are now in clinical use (Hurwitz *et al.*, 2004).

This research has focussed on the possibilities of developing therapeutic interventions targeted at the early stages of colorectal cancer liver metastasis development. Earlier work by Enns et al (Enns *et al.*, 2004;Enns *et al.*, 2005) has identified the integrins, especially the αv subunits, as key components of human HT29 colorectal cancer adhesion within the hepatic sinusoids. Specific targeted therapy, at these molecules, would reduce tumour cell adhesion and subsequent metastasis development. Indeed, the integrins are known to participate in many vital stages in metastasis development (Eble and Haier, 2006) and so antagonists of specific integrins have been evaluated as novel anti cancer agents (Kerr *et al.*, 2002). Vitaxin (Posey *et al.*, 2001;Patel *et al.*, 2001) and cilengitide (Eskens *et al.*, 2003;Smith, 2003) are being tested as future anti cancer treatments. S247 (Reinmuth *et al.*, 2003), an αvβ3 antagonist, has been shown both to decrease colon cancer liver metastases and angiogenesis in mice and to improve survival. Inhibition of α5β1 function with a small peptide (ATN161) in combination with flurouracil infusion has reduced colorectal liver metastases and improved survival in mice (Stoeltzing *et al.*, 2003).

Chapter 6 - *Conservation of Key Integrins In Different Species of Colorectal Cancer,* and Chapter 7 - *Integrin facilitate DHDK12 rat colonic cancer cell migration,* have focused on the role of specific integrin subunits, α2, α6 and β4, in tumour cell migration and extravasation.

Colorectal cancer cell migration from the hepatic sinusoids into the liver parenchyma is a crucial step in the development of liver metastasis (Haier *et al.*, 2003;Enns *et al.*, 2004;Enns *et al.*, 2005). This is believed to swiftly follow colorectal cancer cell targeting and adhesion (Haier *et al.*, 2003;Enns *et al.*, 2004;Gassmann *et al.*, 2004;Enns *et al.*, 2005). Successful extravasation confers many advantages on the tumour cell. Escaping the circulation protects the cancer cells from haemodynamic stresses (Brooks, 1984). Physiological shear stress can also affect the adhesive bonds of the colorectal cancer cells within the hepatic circulation and prevent sustained adhesion. If stabilisation and sustained adhesion can not be achieved, the initial bond can break and the tumour cells re enter the circulation (Haier *et al.*, 2003). Many conventional chemotherapy regimes (Kelly C and Cassidy J, 2007) are administered haematogenously. Concentrations of the cytotoxic drugs remain higher within the circulation than in surrounding tissues. However, current chemotherapy regimes target tumour cells within the mitotic phase. Colorectal cancer cells within the circulation are unlikely to be proliferating, although intravascular proliferation of tumour cells has been seen in some *in vivo* models (Al-Mehdi *et al.*, 2000;Sturm *et al.*, 2003). So, inhibition of migration may not cause a huge increase in the efficacy of conventional chemotherapy regimes.

For metastasis development, extravasation is vital. The colorectal cancer cell migrates from the hostile environment of the circulation into the relative safety of the hepatic parenchyma. Here, the tumour cell can obtain nutrients. The tumour cell can then either remain dormant or interact with local regulatory mechanisms to stimulate self-proliferation and neo-angiogenesis (Chambers *et al.*, 2002;Fidler, 2002).

The three integrin subunits α2, α6 and β4 are known to have a role in extravasation in human HT29 colorectal cancer cell migration (Enns *et al.*, 2004). The initial hypothesis proposed that these subunits may have a key role in early colorectal liver metastasis development from colorectal cancer cells lines other than human HT29. Through the techniques of Immunocytochemistry and Western Blotting, it was shown that these integrins are expressed both in the strain of HT29 and in the rat colorectal cancer cell line DHD K12. This demonstrates conservation of these three integrins in unrelated rat species and also maintained expression of these integrin subunits among the HT29 population. Presence of the integrin subunits α2, α6 and β4 in unrelated colorectal cancer cell lines from different species emphasizes their importance. This importance was further highlighted by our *in vivo* work. Functional blocking of these integrin subunits produced a significant reduction in migration rates and maximum migration rates in those experimental groups.

These findings have important implications. Could these three integrin subunits have a potential role as therapeutic targets in the treatment of early colorectal liver metastasis development?

The α2, α6 and β4 integrin subunits are expressed in colorectal/colonic cancers in two unrelated species. These integrins may be expressed in a wide range of other colorectal

cancers. Highly specific inhibitors of these integrin subunits could act as a vehicle for selective delivery of cytotoxic therapies. This would achieve higher tumour cell mortality with fewer systemic side effects. Integrins are expressed in a wide variety of cells within the human body. Experiments to ascertain which healthy cells express these integrins would need to be performed. Antibodies, specific to the α2, α6 and β4 integrin subunits, could be tagged with a non harmful label and introduced into a rodent host to see where binding occurs. This would identify the tissues and organs where local side effects would occur. The systemic manifestations of this novel therapy could then be calculated.

Functional blocking of specific integrin subunits α6, α2 and β4, although producing different total tumour cells, adherent and extravasated cells visualised, produce very similar migration rates. The very similar migration rates could be attributed to activation of a common downstream pathway or direct interference of the tumour cell antibody complex with the migration process. At least three distinct mechanisms of action have been proposed for integrin participation in tumour cell migration – direct; modification of tumour cell phenotype or enzymatic recruitment and regulation of expression.

The results suggest that the colonic cancer cell antibody has no significant effect on tumour cell adhesion. In the work by Enns et al, antibody inhibition of the αv integrin subunits significantly reduced metastatic cell adhesion (Enns *et al.*, 2005). Enns et al reported that, although absolute numbers of extravasated tumour cells were reduced – secondary to impaired tumour cell adhesion, the relative migration rates were not affected by αv integrin blockade. The tumour cell antibody complex, per se, does not appear to impede migration. To further analyse and clarify the roles of the integrin subunits α6, α2 and β4 in tumour cell migration and completely disprove the theory of

direct antibody interference, knockout mutant models are required. Comparisons of wild type, knockout mutants and mutants with up-regulated expression of a specific integrin subunit with our antibody experimental groups would help to further clarify the mechanism of action. Future work could examine the effect of simultaneously blocking several integrins, known to be involved in colonic cancer cell migration, to investigate a possible synergistic effect. Other colorectal cell lines should be examined to ascertain if these integrin subunits play a more universal role in colorectal cancer cell migration.

Finally, Haier et al (Enns *et al.*, 2004;Enns *et al.*, 2005) showed that, despite blocking multiple cell adhesion molecules, complete inhibition of adhesion was never achieved. Similar findings are likely to be true with regard to extravasation. Many different molecules are likely to be involved in colorectal cancer cell migration.

8.4 Clinical Implications

With best conventional chemotherapy, median patient survival with metastatic colorectal cancer is 24 months (Kelly C and Cassidy J, 2007). The first principle of the modified "seed and soil" hypothesis states that cancers are a heterogeneous population of cells and each subpopulation has its own phenotype (Fidler, 2003). Conventional chemotherapy may be selecting out resistant cancer cells in patients – possibly cancer stem cells (O'Brien *et al.*, 2007). This would account for the fact that patients initially can have a very positive response to treatment, followed by progression of local and metastatic disease. Altering chemotherapy regimes may temporarily hold disease progression in check.

Novel therapies targeted at inhibiting tumour cell inhibition would have significant oncological benefit. 25% of newly diagnosed colorectal cancer patients have established

liver metastases. Inhibition of extravasation would impede tumour cell escape from the circulation. The colorectal cancer cells would have prolonged exposure to physiological shear stress which would prove lethal to many (Brooks, 1984). Inhibition of extravasation would inhibit further metastasis development in patients with established metastasis. More aggressive local oncological management, through surgery or radiofrequency ablation, could be considered routinely.

These integrin subunits α6, α2 and β4 could allow specific targeted therapies to be administered to colorectal cancer cells. Highly selective inhibitors could be used, not only to functionally block these molecules, but to deliver toxic anti-cancer therapies. This would be more efficient and effective than conventional oncological therapies. Such treatment modalities would also significantly reduce the systemic effects that are currently associated with chemo and radiotherapy – effects that can be so severe as to cease treatment tolerance. The specificity of such a system would increase if integral components of the downstream pathways, activated by the integrins, could be identified and targeted as the integrins are expressed in a variety of human cells.

Antibody therapy would have limitations. Exogenously administered antibodies would only have a short therapeutic window before being negated by the immune system. This therapeutic window would decrease with repeated exposure, while the risk of inducing anaphylaxis would increase. The timing of administration and the suitability of the specific cancer to integrin inhibition therapy would be crucial factors. Diagnostic biopsies could be used to ascertain cancer integrin expression. If appropriate, the patient could be selected for integrin inhibition therapy. The two most advantageous times of administration would be at diagnosis or prior to surgery. At diagnosis, antibodies against specific integrins would reduce the risk of metastasis development prior to

further management. Prior to surgery, this therapy would protect the patient from any tumour cells shed into the circulation intra-operatively. Integrin therapies, directed at the early steps of metastasis, are only at an experimental stage. For use in clinical treatment, more research focussed on this area and extensive clinical trials are needed.

8.5 Conclusions

- Integrin subunits α6, α2 and β4 are directly involved in migration of DHDK12 cell line in a Sprague Dawley host.

- Integrin subunits α6, α2 and β4 are expressed in the rat DHDK12 colonic cancer cell line – a novel finding.

- Cancer cell line-host selection has a significant effect on early colonic cancer liver metastasis development. DHDK12-BDIX provides a highly biologically accurate *in vivo* model.

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Appendix 1.

1. Awarded Grants

Aileen Lynn Bequest Fund Grant

Royal College of Physicians and Surgeons Glasgow Jan 2007

2. Publications and Presentations arising from the thesis

John HP Robertson, Shi Yu Yang, Kevin M Sales, Alexander M Seifalian and Marc C Winslet

In Vivo Models for Early Development of Colorectal Liver Metastasis

International J Experimental Pathology 2008 Feb;89(1):1-12.

John HP Robertson, Arthur M. Iga, Kevin M Sales, Alexander M Seifalian and Marc C Winslet

Integrins: A method of early intervention in the treatment of colorectal liver metastases.

Current Pharmacological Design 2008;14(3) in press.

JOHN HP ROBERTSON, ARTHUR M IGA, SHI YU YANG, ALEXANDER M SEIFALIAN AND MARC C WINSLET

A Biologically Accurate *In Vivo* Model for Early Development of Colorectal Liver Metastasis

Submitted to International Journal of Experimental Pathology.

JOHN HP ROBERTSON, ARTHUR M IGA, SHI YU YANG, ALEXANDER M SEIFALIAN AND MARC C WINSLET

Functional Blocking of Specific Integrins Inhibit Colonic Cancer Migration

Submitted to British Journal of Cancer

Published abstracts and presentations to learned societies

John HP Robertson, Arthur M Iga, Shi Yu Yang, Alexander M Seifalian and Marc C Winslet

A biologically accurate *In Vivo* Model for Early Development of Colorectal Liver Metastasis

Poster Presentation

British Microcirculation Society, Queen's University Belfast, 2-3rd April 2007.

John HP Robertson, Arthur M Iga, Shi Yu Yang, Alexander M Seifalian and Marc C Winslet

A biologically accurate *In Vivo* Model for Early Development of Colorectal Liver Metastasis

Microcirculation 2007 Aug; 14(6):635-65.

John HP Robertson, Arthur M Iga, Kevin M Sales, Shi Yu Yang, Alexander M Seifalian and Marc C Winslet

In Vivo Models for Early Development of Colorectal Liver Metastasis

Poster Presentation

Association of Coloproctology GB and Ireland Glasgow Meeting 2nd-5th July 2007.

John HP Robertson, Arthur M Iga, Kevin M Sales, Shi Yu Yang, Alexander M Seifalian and Marc C Winslet

In Vivo Models for Early Development of Colorectal Liver Metastasis

Abstract

Colorectal Disease 2007 Vol 9(1) p62.

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